University of Alberta

An Immunocapture Assay for the Detection of *Mycobacterium avium* subsp. *paratuberculosis* for the Diagnosis of Johne's Disease

by

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Abstract

Paratuberculosis or Johne's disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) is an infectious, progressive chronic digestive disorder of both wild and domestic ruminants and has great economic impact especially in the cattle industry. Due to the absence of a sensitive detection assay for identifying the infected animal at an early phase, the infection can spread silently throughout a herd. Antibiotic treatment is not recommended and culling is the only solution to the problem. In this study, we developed a diagnostic assay that captured and concentrated *Map* using antibody followed by amplification using polymerase chain reaction (PCR) to detect the organisms. Purified antibodies IgY were produced from eggs by injecting *Map* into breast muscles of Single Comb White Leghorn chickens followed by direct coating of the surface of cellulose/iron oxide beads (MagaCell ™-IgY beads) or indirect coating via MagaBeads™ rabbit anti-chicken IgG linker for capturing *Map* from bovine feces. Optimization for the immunocapture was performed with respect to holding time, temperature, volume and types of immunocapture beads. DNA extracted from the captured bacteria by both methods was amplified by PCR to determine the sensitivity and specificity of the assay. MagaCell ™-IgY beads proved to be better than indirect capture and were selected for the field study using bovine feces from positive and negative Johne's disease farms. There was 100% specificity and the sensitivity by real-time and conventional PCR after immunocapture and immunocapture culture were 88.6%, 77.3% and 61.4% respectively. In the category of heavy to moderate growth of *Map* colonies on culture slants, the sensitivities of real-time PCR and conventional PCR

after immunocapture and immunocapture culture were 94.4%, 88.9%, 94.4% but the sensitivities dropped to 84.6%, 69.2% and 38.5% in the category of scant to light growth by culture. Conventional PCR was less sensitive than real-time PCR and inhibition was observed in field samples with conventional PCR after immunocapture. By using this immunocapture assay in combination with front-end automation for DNA extraction and real-time PCR, 50 samples can be processed within an eight hour shift by one person. This rapid turn-around time for reporting is a great improvement over conventional fecal culture that requires 8 to 16 weeks. Therefore this immunocapture-PCR assay has great potential as a routine test for *Map* in bovine feces for Johne's disease in a diagnostic laboratory.

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I dedicate this thesis to my Beloved Mother who is watching over me from heaven.

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List of Abbreviations

A	adenine
ADC	Albumin Dextrose Catalase
AGID	agar gel diffusion test
ATCC	American Type Culture Collection
BCG	Bacillus Calmette Guérin
bp	base pair
BHI	Brain Heart Infusion
C	cytosine
CF	Complement fixation
CSPD	(disodium3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate
DNA	deoxyribonucleic acid
DIG	digoxigenin
dNTP	deoxynucleotide triphosphate
ELISA	enzyme-linked immunoadsorbent assay
fg	femtogram
G	guanine
g	gram(s)
h	hour(s)
HEYM	Harrold Egg Yolk Medium
HEYMX	Herrold's egg yolk medium without mycobactin

HPC-BHI	1-Hexadecylpyridium chloride-Brain Heart Infusion
HRPO	horseradish peroxidase
kb	kilobase
kDa	kilo dalton
IF	immunofluorescent
IC	internal control
IMS	immunoseparation
l	liter
LAM	lipoarabinomannan
LB	Luria Bertani
LC	lightcycler
M	molar
Mb	Megabase
Map	*Mycobacterium avium* subsp. *paratuberculosis*
$MgCl_2$	magnesium chloride
min	minute
mg	milligram
ml	milliliter
mM	millimolar
µg	microgram
µl	microliter
µm	micrometer

µM	micromolar
ng	nanogram
OD	optical density
pg	picogram
PCR	polymerase chain reaction
PPA	protoplasmic antigen
PBS	phosphate buffer saline
PCR	polymerase chain reaction
RJT	rapid Johne's test
RNA	ribonucleic acid
RFLP	restricted fragment length polymorphism
SCWL	Single Comb White Leghorn
SSC	Sodium chloride-Sodium citrate
T	thymine
TAT	turn-around time
TMB	3,3' 5,5' Tetramethybenzidine
s	second(s)
subsp.	subspecies
SDS	sodium dodecyl sulfate
U	unit
Z-N	Ziehl-Nielsen

1. Introduction

Mycobacterium avium subsp *paratuberculosis* (*Map*) is the etiological agent for Johne's disease, a chronic digestive disorder of both wild and domestic ruminants that occurs worldwide. It has great economic impact due to financial loss of culling the infected aniamls, death of diseased animals, reduction in reproductive efficiency and milk production in subclinically infected animals. The infected animal can be asymptomatic for a long time while shedding the infectious bacteria into the surroundings and infecting more animals. The most susceptible age groups for infection are newborn and young calves. In recent years, rabbits and foxes have been found to be infected and they can act as vehicles of transmission because they move from farm to farm. Although conventional culture for *Map* in bovine feces has been the gold standard for the detection of *Map* infection, the turn-around time (TAT) for reporting ranges from 8 to 20 weeks depending on the number of organisms being shed by the animal at that particular stage of the disease.

Enzyme-linked immunosorbent assay (ELISA) is an alternative method for clinical diagnosis of Johne's disease. However, the sensitivity varies depending on the level of humoral response in the infected animal. Molecular testing by PCR seems to be more sensitive but fecal specimens are complex and heterogeneous and they contain nonspecific inhibitors that are coextracted with target DNA and interfere with the amplification assay. The objective of this study is to develop a diagnostic assay, which would provide good sensitivity, specificity and rapid TAT for the detection of *Map*.

The objectives of this research were divided into six phases and they were to:

1. Compare different DNA extraction methods for performing *Map* PCR;
2. Construct an internal control to monitor the presence of inhibitors;
3. Produce and purify polyclonal antibodies from eggs;
4. Optimize the immunocapture assay;
5. Compare conventional and real-time PCR after immunocapture;
6. Validation trials with bovine feces.

2. Literature review

2.1 Paratuberculosis

2.1.1 *Discovery and morphology*

The etiological agent for paratuberculosis or Johne's disease is *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), initially reported by H.A. Johne and L. Frothingham in Germany in 1895 when acid-fast bacilli were found in the histological sections of intestine from cattle with enteritis. However, it was not until 1910 that F. W. Trowt was able to grow the bacteria in the laboratory and induced infection in experimental cattle (Chiodini *et al.*, 1984).

Map belongs to the genus *Mycobacterium*, a relative of of the genera *Corynebacterium*, *Nocardia*, *Rhodococcus* and *Streptomyces*. Mycobacteria can be divided into two major clusters based on their growing characteristics. The fast growers are usually consisted of the nonpathogenic environmental isolates and the slow growers are respresented by pathogens such as *M. avium*, *M. bovis*, *M. leprae*, *M. tuberculosis*, and *Map*. Analysis of the rDNA genes of mycobacteria further confirms the division of this genus into two separate clusters. The fast growers contain two sets of rRNA genes whereas only one copy is present in the slow growers (Bercovier *et al.*, 1986, Chiodini, 1989, 1990,). The rDNA genes are found in a single operon in *Map* (Frothingham, 1999, Liesack *et al.*, 1990, 1991) and it does not contain a tRNA gene. An internal transcribed spacer region of approximately 280 bp separates the 16 and 23S rDNA and

another 91 bp separates the 23S rDNA and 5S rDNA genes (Liesack *et al.*, 1991). Both *Map* and *M. avium* have an unusal insertion of approximately 16 nucleotides in the 23S rRNA gene, which is also found in the 23S rRNA genes of other mycobacteria (Liesack *et al.*, 1991, van der Giessen *et al.*, 1994).

M. avium and *M. intracellulare* have been referred to as the *M. avium* complex. Based on DNA–DNA hybridization studies, *M. avium* can be subclassified into *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis* (*Map*), and *M. avium* subsp. *silivaticum* (*M. silivaticum*). The base composition of *Map* DNA is 66 to 67% G+C (Imaeda *et al.*, 1988, McFadden *et al.*, 1987) as compared to 66 to 68% for *M. avium* (Clark-Curtiss *et al.*, 1990).

The size of the Map genome is 5.48 Mb and has been completely sequenced at the University of Minnesota, St Paul in collaboration with the National Animal Disease Center (NADC: Ames, Iowa). The genome sequence was released January 2004 (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=41400296, accession number AE016958). *Mycobacterium avium* has a genome of 4.83 Mb and is highly homologous to *Map* with a homology of greater than 97% in a 35-Kb region encompassing the origins of replication of *Map* and *M. avium* (Bannantine *et al.*, 2003).

Map, when observed under the microscope, they are slightly bent or curved slender rods (2 to 4 μ long and 0.2 to 0.5 μ wide), pleomorphic and may undergo branching or filamentous growth. The organism is a slow grower with a generation time of 20 h and depends on mycobactin for growth. The dependency on mycobactin for growth also differentiates *Map* phenotypically from *M. avium* and *M. silivaticum*. On

solid agar, the colony morphology tends to be wrinkled, compact and the bacteria have the tendency to pile up rather than spreading out over the surface of the agar. The cell wall of these bacteria has a high lipid content resulting in hydrophobic character and they adhere to each other during growth. The lipid component is made up of mycolic acids that are relatively impermeable to various basic dyes. Once stained, they retain the dyes and resist decolorization with acidified organic solvents, therefore *Mycobacterium* species are classified as acid fast. This lipid rich cell wall also contributes to resistance to killing by acid and alkali and the slow growing characteristics may be due to low permeation of nutrients into the cell.

2.1.2 *Insertion elements*

Several insertion elements IS*900*, IS*1311* and IS*1245* have been found in the *Map* genome. IS*900* is a 1451-1453 bp repetitive element and was the first insertion sequence identified in mycobacteria (Collins *et al.*, 1989, Green *et al.*, 1989) and are present in 15 to 20 copies in the *Map* genome. One of the unique features with this insertion element is the lack of terminal inverted repeats. The IS*900* element seems to be unique to *Map* and has been widely used as a diagnostic tool to detect *Map* in clinical samples, yet it has an 82% homology with IS*1626*, an insertion element identified in the *M. avium* genome (Harris and Barletta, 2001). IS*1311* is also present in *M. intracellulare* and IS*1245* is present in *M. avium* isolates. Another insertion element IS*1612*, with a size of 7 Kb is found in both *Map* and *M. avium* that may be coding for biosynthesis, modification and transfer of a fucose molecule to cell wall

glycopeptidolipids (Sheridan *et al.*, 2003). The function of these insertion elements may contribute to the regulation of gene expression (Bull *et al.*, 2000).

2.1.3 *Proteins and antigens*

Several proteins found in *Map*, which include two heat-shock proteins GroES and GroEL that have homology to other mycobacterial antigens. The GroES, approximately 100 amino acids in size, is highly conserved, and found in *Map* and *M. avium* (Cobb and Frothingham, 1999; El-Zaatari *et al.*, 1995). Its amino acid sequence shares over 90% identity to the GroES protein found in *M. tuberculosis* and *M. leprae*. The GroEL antigen is a 65-kDa protein, which is highly immunogenic and homologous to similar proteins (Shinnick, 1987; Thole *et al.*, 1987, 1988) identified in *M. tuberculosis*, *M. leprae* and *M. avium*. Other immunoreactive proteins of *Map* include a 32-kDa secreted protein with fibronectin binding properties (Andersen *et al.*, 1991; El-Zaatari *et al.*, 1994) and a 34-kDa cell wall antigenic protein (de Kesel *et al.*, 1992). This 34-kDa protein is homologous to a similar protein found in *M. leprae* (Silbaq *et al.*, 1998) and shares two identical B-cell epitopes with the *M. avium* homolog (Ostrowski *et al.*, 2003). A 34-kDa protein with putative serine protease activity has been described (Cameron *et al.*, 1994) and is different from the 34-kDa antigen described above. Alkyl hydroperoxide reductase proteins (AhpC and AhpD), likely involved in the detoxification of reactive nitrogen intermediates, are found in abundance in *Map* (Olsen *et al.*, 2000) compared with *M. avium* when examined under various laboratory conditions (Olsen *et al.*, 2000). Other proteins such as the 32-kDa

antigen (El-Zaatari *et al.*, 1994), 16.7-kDa antigen, manganese-dependent superoxide dismutase (SodA) and proteins of the antigen 85 complex (Dheenadhayalan *et al.*, 2002; Liu *et al.*, 2001; Mullerad *et al.*, 2003), elicit T helper 1 (Th1)-type immune responses associated with protective immunity in mice.

A *Map*-specific antigen is the HspX protein, a 16-kDa putative heat-shock-like protein for mediating cell attachment and stimulating phagocytosis (Ellingson *et al.*, 1998). This protein is expressed by intracellular *Map* within bovine macrophages (Bannantine and Stabel, 2000). The Hed (host-expression-dependent) protein (Doran *et al.*, 1997) is encoded by IS*900* and it is unclear whether this protein plays a role in elicits an immune response. In addition, a 35-kDa membrane protein that reacts with antisera from cattle with Johne's disease also plays a role in the invasion of bovine epithelial cells (Bannantine *et al.*, 2003). The major antigen in mycobacterial species including *Map* is lipoarabinomannan (LAM), which has been used in many serological tests for diagnosis of Johne's diseases (Sugden *et al.*, 1987). Other lipids and glycolipids of *Map* have not been fully characterized but they are expected to be similar to the ones found in other mycobacterium species. A summary of representative proteins and antigens is shown in Table 2.1.

Table 2.1. Representative proteins associated with *Map*

Antigens	Characteristics	Function	Reference
Lipoarabinomannan (LAM)	Cell wall component	Serological test for Johne's disease	Sugden *et al.*, 1987
GrosES GrosEL 65-kDa	Heat shock protein	Highly antigenic shares homology with other mycobacterial antigens	Cobb *et al.*, 1999; El-Zaatari *et al.*, 1995
AhpC and AhpD	Alkyl hydroperioxidase reductase proteins	Detoxification of reactive nitrogen intermediates	Olsen *et al.*, 2000
34-kDa (Antigen D)		Serine protease activity	de Kesel *et al.*, 1992
34-kDa	Cell wall antigen	Elicit humoral response	Cameron *et al.*, 1994
32-kDa antigen	Immunogenic protein		El-Zaatari *et al.*, 1994
16.7-kDa	Immunogenic protein	Elicit T helper 1 type immune response	Mullerad *et al.*, 2003
SodA	Immunogenic protein	Manganese-dependent superoxide dismutase	Liu *et al.*, 2001
85 complex	Immunogenic protein	Elicit T helper 1 type immune response	Dheenadhayalan *et al.*, 2002
44.3-kDa	Soluble protein		Mutharia *et al.*, 1997
34.5-kDa	Soluble protein		Mutharia *et al.*, 1997
P35 antigen	Shared with other *M. avium* species		El-Zaatari *et al.*, 1997
P36 antigen		Indication of Johne's disease	El-Zaatari *et al.*, 1997
36 kDa (FAP-P)	Fibronectin binding protein	Located in the inner part of the cell envelope	El-Zaatari *et al.*, 1994
HspX protein	Heat-shock-like protein	Mediating cell attachment and stimulating phagocytosis	Ellingson *et al.*, 1998

2.1.4 *Disease prevalence and economic impact*

Paratuberculosis is found worldwide and has been considered one of the most serious diseases affecting dairy cattle with significant impact on the global cattle economy (Chiodini *et al.*, 1984; Merkal *et al.*, 1987; Pandey *et al.*, 1987). The annual loss due to this disease in the US was estimated to exceed $200 million to $1.5 billion (Jones 1989; Stabel *et al.*, 2004). An estimated loss of US $227 to $245/cow/year was reported (NAHMS, 1997; Ott *et al.*, 1999) in dairy herds with clinical Johne's disease. The financial loss was mainly due to culling, death of the diseased animals, reduced reproductive efficiency and reduced in milk production in subclinically infected animals. A 15-16% reduction of milk production in subclinically infected animals accounted for the major loss to the dairy industry (Abbas *et al.*, 1983; Benedictus *et al.*, 1987; Johnson-Ifearulundu *et al.*, 1997).

In a survey at a Pennsylvania slaughterhouse (Cocito *et al.*, 1994), the prevalence of Johne's disease was 7.2% in dairy cattle. Similar results were reported in Alberta (Sorenson *et al.*, 2003), Wisconsin (Collins *et al.*, 1994) and Michigan (Johnson-Ifearulundu, 1999). The National Animal Health Monitoring System (NAHMS) Dairy 1997 study in the US reported about 40.6 ± 2.0% of the dairy operations had at least 1 *Map* seropositive cow while 16.8 ± 1.5% had two or more seropositive cows. Van Leeuwen and co-workers (2001) reported 43.3 ± 10.4% of dairy herds in Maritime Canada had at least 1 cow that was seropositive for *Map*, while 16.7 ± 7.9% had two or more seropositive cows. A recent study (Adaska and

Anderson, 2003) has shown the seroprevalence of *Map* in dairy cattle in northern, central and southern California to be at 14.1, 7.5 and 10.6%, respectively.

2.1.5 *Transmission and natural history of the disease*

The transmission of Johne's disease is by the fecal-oral route, with bacteria ingested from contaminated pasture, soil, water or contaminated surfaces. Calves can also be infected by ingestion of colostrum and milk from an infected cow (Chiodini *et al.*, 1984; Sweeney *et al.*, 1992) and transmammary shedding of the organisms has been reported in 12% of cows with subclinical infection (Sweeney *et al.*, 1992). In cattle, the infection is usually contracted in the first few months of life (Stabel *et al.*, 1998) and there is a delay in clinical manifestation from 6 months to 15 years (Chiodini *et al.*, 1984; Manning and Collins, 2001). Newborn calves are more susceptible to the disease than older animals probably due to the immaturity of the immune system (Clarke *et al.*, 1994; Cocito *et al.*, 1994; Larsen *et al.*, 1975). Adult animals can be infected, but they require a higher infectious dose and have a longer incubation period compared with younger animals (Whitlock and Buergelt, 1996). Cattle as young as 13 to 14 months of age shed *Map* bacteria during experimental as well as in natural infection (de Lisle *et al.*, 1980). Cattle shedding *Map* are potential vehicles for horizontal transmission especially when heifers are housed in groups under unsanitary conditions. The number of viable bacilli present in the feces of a clinical case is estimated to be between 10^6 to 10^8 bacteria/g (Jorgensen 1982). Due to the prolonged nature of the disease, persistent

infection occurs in the population by spreading from the older animals to the susceptible calves, especially newborns.

Intrauterine infection in cattle (Seitz *et al.*, 1989) seems to be confined to fetuses of cows in an advanced stage of disease. The major veterinary focus of this disease is cattle but infections among other animal species such as deer (Riemann *et al.*, 1979), fox, stoat (Beard *et al.*, 1999), rabbits (Greig *et al.*, 1997; 1999), goats (Lenghaus *et al.*, 1977; Bauerfeind *et al.*, 1996; Sigurðardóttir *et al.*, 2001), sheep (Seaman *et al.*, 1981; Bauerfeind *et al.*, 1996; Gwozdz *et al.*, 2000) and alpaca (Ridge *et al.*, 1995) have been reported.

This disease can be divided into three clinical stages. Stage 1 is the initial subclinical infection with *Map* undetectable in feces, low humoral and high cellular immune response. Stage 2 is the subclinical and excretory phase in which there are no obvious symptoms, the number of the bacteria increases in the intestinal lumen and mucosa and *Map* cells can be found in the feces. Stage 3 is the clinical and excretory phase in which presentation is characterized by chronic diarrhea and symptoms of generalized infection and *Map* cells are shed in the feces in high number. Emaciation, decreased milk production, diffuse edema, anemia and infertility are the dominant features of this stage. The diseased animal is rarely treated and eventually dies as a result of cachexia. Other observations associated with the infection include increased incidence of mastitis and reproductive disorders resulting in a prolonged calving period compared with a healthy animal (Abbas *et al.*, 1983; Buergelt and Duncan, 1978; Merkal *et al.*, 1975). Abortion is rarely seen even in the advanced stage of the disease.

The manifestations of the disease can last from 6 months to 15 years (Chiodini *et al.*, 1984; Manning *et al.*, 2001).

2.1.6 *Pathogensis of paratuberculosis*

The route of entry is by penetration of the M cells located in the dome epithelium covering the ileal Peyer's patches in the small intestine. The organisms are phagocytosed by the subepithelial and intraepithelial macrophages and they multiply intracellularly (Momontani *et al.*, 1988). Lesions are formed as small granulomas develop in the interfollicular and basal region of the Peyer's patches and eventually spread to the adjacent lamina propria and villi. This localized infection develops into a generalized infection with the multiplication of the bacteria in the nearby lymph nodes (Momotani *et al.*, 1988; Zurbrick *et al.*, 1987). This results in a chronic catarrhal inflammation along with hyperplasia and intense histiocyte infiltration of the lamina propria (Merkal *et al.*, 1970). Hypoproteinemia develops due to the diffuse granulomatous reaction along with edema and the wall of the gut thickens progressively. Eventually the epithelial cells of the mucosa are sloughed into the intestinal lumen and result in the discharge of a large number of bacterial cells at a concentration estimated at 10^6 to 10^8 viable organisms/g of feces (Jorgensen *et al.*, 1982). This massive discharge of bacteria into the environment may account for the rapid spread of the infection even from a single infected animal to a herd of susceptible animals.

2.2 Diagnostic tests for the detection of *Map*

2.2.1 *Microscopic examination*

Microscopic examination of bovine fecal specimens by acid fast or fluorescence staining can detect the presence of the organisms but it fails to differentiate environmental mycobacteria from *Map*, the infectious agent. Furthermore, the number of organisms found in the stool varies depending on the stage of the disease. Therefore, this technique is not appropriate especially in the initial subclinical phase.

2.2.2 *Culture techniques*

Culturing *Map* from feces is more sensitive than microscopic examination but it requires a lengthy and tedious procedure for the decontamination process to eliminate faster growing microorganisms other than *Map* and requires 8 to 20 weeks of incubation. Presently, Herrold Egg Yolk Medium (HEYM) is widely used for the isolation of *Map*. Studies on other components of the growth medium used to facilitate the culture of *Map* includes mycobactin, iron and protein sources from serum, lymph nodes and eggs (Merkal and Curran, 1974; Merkal *et al.*, 1964, Merkal and Larsen, 1962). Rapid detection of mycobacteria from humans and bovine origin was facilitated through the use of radiometric culture with BACTEC 460 TB system (Becton Dickinson) in which a ^{14}C-labelled substrate (often palmitic acid) is used as the carbon source in a liquid medium. This labelled carbon is metabolized to radiolabeled carbon dioxide that can be measured in the gas phase in the vial (Reggiardo and Tigertt, 1977; Yearsley *et al.*,

1998). However, the use of radioactive substrate is a disadvantage due to several reasons. The technical staff require special training for handling radioactive materials, careful monitoring of both radiaoactive wet and dry waste and their disposal. With the BACTEC 9000 MB system (Becton Dickinson), bacterial growth is measured through fluorescence of a ruthenium metal sensor impregnated in silicon rubber contained within the vial of the medium. Fluorescence of this sensor is a function of the oxygen partial pressure. Decrease in the oxygen pressure caused by bacterial growth results in an increase in fluorescence units. The application of radiometric culture for *Map* (Damato and Collins, 1990; Collins *et al.*, 1990) has improved the reporting time from weeks to approximately 9 days; however, detection is dependent on the bacterial load of the specimen and stage of the disease.

Fecal culture is most effective in detecting diseased cattle in later stages of infection when the animal is shedding very high numbers of bacteria (Sanftleben 1990; Whitlock *et al.*, 2000). Less than ten organisms per gram of feces were detected using the radiometric technique with filter concentration (Collins *et al.*, 1990). The detection level was 1000 organisms per gram of feces by conventional culture with a sedimentation technique (Whipple and Merkal, 1985). Culture technique is still considered to be the most reliable test for detecting subclinically infected animals. It remains the "gold standard" for the identification of *Map* from tissue and fecal specimens (Chiodini *et al.*, 1984; Colgrove *et al.*, 1989). It is used as the standard for comparison of all new tests (de Lisle *et al.*, 1980) and serves as the basis for management decisions of veterinarians and farmers (Thoen and Baum, 1988).

To isolate *Map* from clinical specimens, specifically fecal material, decontamination is an absolute requirement to prevent over growth of other bacteria from the gut flora. During this process, the number of viable organisms in the specimen may be reduced by exposure to disinfectants or antibiotics or by centrifugation or sedimentation. A balance is required in the decontamination step to ensure that the target bacteria are not killed (Eamens *et al.*, 2000; Stabel, 1997; Whipple *et al.*, 1992). Studies have shown the estimated loss of mycobacteria during the decontamination process to be about one to two logs using various culture techniques for *Map* (Jorgensen, 1982; Merkal, 1973; Whitlock and Rosenberger, 1990). A similar study also showed the loss of *Map* throughout the decontamination protocol for fecal and tissue samples isolated by the BACTEC culture method (Reddacliff *et al.*, 2003). There are contradictory reports regarding the viability of the organism during the decontaminating process (Mokresh *et al.*, 1989). There are various methods of reducing bacterial and fungal contamination to improve the recovery of *Map* including treatment of specimens with oxalic acid, NaOH, sodium hypochlorite, phenol, benzalkonium chloride and 0.9% HPC (hexadecylpyridinium chloride) with the latter two being widely used (Merkal *et al.*, 1982; Turcotte *et al.*, 1986). In 1993, this modification was adopted by the Animal Health Service in the Netherlands as a routine test for bovine paratuberculosis. Unfortunately, no published studies were available comparing this protocol with the culture method commonly used in USA (Stabel, 1997).

To increase the sensitivity of a culture method, the incubation period can be extended from 8 to 20 weeks but any further extension after that does not influence the

outcome. A concentration step using high-speed centrifugation yields a higher number of organisms per inoculum and allows an earlier detection of *Map*. However, there is a lack of a standardized cultivation procedure for *Map* worldwide (Jorgensen, 1984, Kim *et al.*, 1989). Therefore the results submitted from individual diagnostic laboratories may not be an accurate estimate of infection in the herds because this figure will depend on the rate of recovery of the organisms with the technique used in that particular laboratory. Whipple *et al.* (1991) published a review of culture isolation protocols of *Map* from fecal specimens and suggested a standardized cultivation procedure.

2.2.3 *Histological examination*

Histopathological analysis of the terminal ileum and mesenteric lymph nodes has been done on biopsy material from cattle infected by *Map*. The presence of mycobacterial clumps of cells in granulomatous tissue is used as confirmation of infection.

2.2.4 *Immunological assays*

Immunological assays such as complement fixation test (CF), agar gel immunodiffusion assay (AGID) and enzyme-linked immunoadsorbent assay (ELISA) are commonly used to diagnose paratuberculosis in a herd (Colgrove *et al.*, 1989). The close genetic relatedness of *Map* to the other mycobacterium species, especially with other strains of the *M. avium* complex, presents an obstacle to the development of serological assays for Johne's disease (Camphausen *et al.*, 1988; McIntyre and Stanford

1986). Comparable studies of these tests show discrepancies in the ability to identify the infected animals (Darcel, 1995; Darcel and Logan-Handsaeme, 1998; Hilbink *et al.*, 1994; McNab *et al.*, 1991). This may be due to the absence of the entire range of immunodominant antigens for *Map* within a given test (Sugden *et al.*, 1997). Harris *et al.* (2001) published a detailed summary of the performance of different serology tests correlated with the stage of Johne's disease and infection status. This summary clearly illustrated that the sensitivity of these tests varied with the stage of the disease.

2.2.4.1 *Complement fixation (CF) test*

This test was among the first serological screening test for Johne's disease in the international export and import of cattle (Colgrove *et al.*, 1989). However, it has a low sensitivity and failed to detect infected animals especially in the early stage of the disease (de Lisle *et al.*, 1980; Whitlock, 1991). Furthermore, there is no standardization in the preparation of the crude carbohydrate antigen among the different countries using this test, making interpretation of the data difficult.

2.2.4.2 *Agar gel diffusion test (AGID)*

Agar gel diffusion test is commercially available and is also known as Rapid Johne's test (RJT). It is a serological testing for detecting the presence of antibody to prepared *Map* antigen. It has great diagnostic value with a sensitivity ranging from 54 to nearly 100% (Sherman *et al.*, 1984, 1989, 1990) in the diagnosis of Johne's disease in cattle providing the animal is showing clinical symptoms of weight loss or diarrhea.

However, it fails to detect asymptomatic animals that have a positive culture. Because there is no standardization of the protein used as antigen for AGID test in Canada, USA (McNab *et al.*, 1991, Sherman *et al.*, 1984) and the European countries (Goudswaard *et al.*, 1976), comparison between these jurisdictions is not reliable.

2.2.4.3 *Enzyme-linked immunosorbent assay (ELISA)*

Many variations of the ELISA method have been used for the detection of *Map* antibodies (Cox *et al.*, 1991; Jark *et al.*, 1997; Sweeney *et al.*, 1994) and one of the key components of the assay is the preparation of antigens to capture the antibody from the serum sample of the test animal. The preparation of the antigen for ELISA directly affects the sensitivity and specificity of the assay. A study on three commercial kits showed cross-reactivity between the protoplasmic antigen (PPA) and the lipoarabinomannan (LAM) polysaccharide antigen with *Map* strain 18 but not with strain VRI 316/102-2 (Sugden *et al.*, 1997). The cross-reactivity clearly indicated the sharing of some common antigens in these tests but the lack of cross-reactivity between PPA and LAM ELISA revealed the absence of shared antigens in these preparations. These data showed that the commercially available ELISA test might capture only a subset of the strains infecting the animals. Preabsorption of test sera with antigens of *M. phlei* (Bech-Nielsen *et al.*, 1992 a, b and c; Collins and Sockett, 1993) improved the ELISA test. This step removes nonspecific antibodies against mycobacteria that could cross react with *Map* antigens. An enzyme immuno assay (Billman-Jacobe *et al.*, 1992; McDonald *et al.*, 1999) for gamma interferon has been subjected to comparison studies

in cattle with paratuberculosis. Due to cross reactivity between the mycobacterium species, a positive response for the above tests may not be a true indication of disease (McDonald *et al.*, 1999).

The sensitivity of ELISA in cattle was reported to be 87% in clinical cases compared with 75% in subclinical, heavy fecal shedders of *Map* and 15% in subclinical, light fecal shedders of *Map* (Sweeney *et al.*, 1995). In a mixed herd of subclinically infected and non-infected cattle, the herd-level sensitivity of ELISA is about 45% (Collins and Sockett, 1993) and 45 to 55% in fecal culture (Sockett *et al.*, 1992), respectively. In an analysis by Whitlock *et al.*, (1999) the sensitivity for both fecal culture and ELISA in cattle was reported to be about 35%. Once again, the sensitivity depends on the stage of the disease whether the humoral response of the infected animal is present or absent.

2.2.4.4 *Molecular testing*

The discovery of specific repetitive DNA insertion sequences provided a useful probe to test for the presence of *Map* DNA in fecal samples. Although this sequence is present in multiple copies per organism, the detection level is far less sensitive than the fecal culture method that can detect 10 to 100 organisms/g of feces (Whitlock and Rosenberger, 1990; Whipple *et al.*, 1992). Consequently, amplification techniques such as PCR have been developed for *Map* to increase the sensitivity of detection; however, there are unknown inhibitory factors in clinical specimens that co-extract with the target nucleic acid and interfere with the PCR (Al-Soud and Radstrom, 2000; Wilde *et al.*,

1990). Therefore, it is very difficult to perform PCR directly on fecal specimens and initial culture is required to circumvent this problem.

Radiometric culture, a modification of the standard culture technique, was successfully combined with IS*900* PCR analysis to obtain relatively rapid confirmation of the presence of *Map* (Cousins *et al.*, 1995). This method involved the re-inoculation of a primary radiometric culture containing egg yolk to a similar culture medium without egg yolk after a certain growth index was obtained. The growth index is the correlation of bacterial growth and the generation of fluorescence units generated in the BACTEC 9000 MB system (Becton Dickinson) that was predetermined by the company. A PCR assay was performed on the extracted DNA from the second culture to avoid inhibitors from the egg. This method was also applied to feces, mesenteric lymph nodes and intestinal tissue samples from cattle, sheep and goats with Johne's disease. Whittington *et al.* (1998) managed to eliminate the subculturing from the primary culture by differential centrifugation, the organisms were lysed by heat and the DNA was then used in the PCR. If the test was negative, the DNA in the lysate was further purified with guanidine thiocyanate and silica. Cultures from fecal and ileal samples and lymph nodes from cattle, sheep and goats known to have or suspected of having Johne's disease yielded positive PCR results from 1 to 7 weeks after inoculation. A similar result was obtained with soil and pasture samples that had been spiked with *Map*. The result from this combination of culture and PCR techniques proved to be more sensitive than histopathological examination or culture on Herrold's egg-yolk medium for the detection of infection.

A diagnostic PCR test for Johne's disease based on IS*900* was developed commercially by Idexx (Vary *et al.*, 1990) with a detection level at 10^4 organisms/g of feces (Whipple *et al.*, 1992). There was inconsistency in reporting the sensitivity of the assay between different investigators (Collins and Sockett, 1993; Whipple *et al.*, 1992) and reproducibility was not achieved when low numbers of organisms were present in the feces. The uneven distribution of organisms throughout a fecal specimen or the presence of nonspecific inhibitors co-purified with the DNA could be contributing factors. Another PCR test for Johne's disease based on the ribosomal RNA gene was reported (van der Giessen *et al.*, 1992) but it lacked specificity due to cross-reactivity with some other slow-growing mycobacteria of the *Mycobacterium avium* complex which have similar sequences.

2.3 Control of paratuberculosis

2.3.1 *Reservoir for Map*

It is important to understand the mode of transmission of *Map* and to identify the source of infection before developing an effective infection control program. The principal transmission route of *Map* is most likely due to the contamination of the environment from manure of the infected animals. *Map* remained viable up to 250 days in water and feces (Berg-Jorgensen, 1977; Harris *et al.*, 2001). In a recent study by Whittington *et al.* (2004), *Map* was found to survive up to 55 weeks in a dry fully shaded environment. This environment will provide the reservoir for the spread of the infection to susceptible animals and because the nature if this disease is chronic it is

easily spread to the rest of the herd or herds newly introduced onto the farm. Vertical transmission during pregnancy has also been reported because *Map* has been isolated from the uterus (Kopecky *et al.*, 1967), fetal tissue (Lawrence, 1956) and semen (Larsen and Kopecky, 1970).

Map has a very wide host range from ruminants to monogastrics such as pigs (Thoen *et al.*, 1975) and rabbits (Greig *et al.*, 1997). In North America, *Map* has also been isolated from wild animals such as white-tailed deer (Chiodini and Van Kruiningen, 1983; Shulaw *et al.*, 1986), mule deer (Williams *et al.*, 1983a, b), bighorn sheep (Williams *et al.*, 1983a, b), Rocky Mountain goats (Williams *et al.*, 1983a, b), bison (Buergelt *et al.*, 2000) and elk (Williams *et al.*, 1983b). It has also been found in wild red deer from the Italian Alps (Nebbia *et al.*, 2000) and wild rabbits in Scotland (Angus, 1990; Greig *et al.*, 1997, 1999). Johne's disease has been detected in domestically raised deer (de Lisle *et al.*, 1993; Godfroid *et al.*, 2000; Power *et al.*, 1993), alpacas (Ridge *et al.*, 1995) and elk (Jessup *et al.*, 1981; Manning *et al.*, 1998). Williams *et al.* (1983a) have shown that a strain of *Map* that originated from Bighorn sheep was able to infect other species of wild animals as well as domestic ruminants. This same study also showed that healthy elk and sheep were infected when exposed to diseased animal or contaminated pen effluent. The potential to spread the disease between the domestic and wild animals poses a problem for relocation of animals into the wild or introduction of new livestock onto farmlands previously inhabited by infected herds.

2.3.2 *Control management*

Control programs for Johne's disease have been established in a number of countries. The main goal is to prevent the spread of the disease through the sale of animal replacement stock from infected herds. Control of the disease in cattle herds involves hygienic measures on the farms and also separation of the calves from the herd especially away from the animals that are excreting the organisms (Abbas *et al.*, 1983; Chiodini *et al.*, 1984). The most important on-farm management practices are overall cleanliness, manure handling, new-born calf care and restricted contact between calves and adult animals. ELISA serology and fecal culture are most commonly used for screening herds. The United States has initiated a voluntary 4-stage Johne's Disease herd Status Program for cattle in an effort to identify herds that test negative for *Map* and those herds with a reduced risk of being *Map* infected. Australia has the Market Assurance Program to identify *Map*-free herds to control the movement of cattle from infected to disease-free areas.

2.4 Treatment

There is no consensus on drug treatment for Johne's disease in livestock. Antibiotic treatment is expensive and not very affective due to the inability of the drug to contact *Map* which is usually located intracellularly (Cocito *et al.*, 1994). Antibiotic therapy is only applicable to very valuable livestock and is used to prolong the animal's life for breeding purposes. There are different reports on the use of conventional

antituberculous and anti-leprosy drugs in the treatment of *Map* infected in cattle, sheep, goats and rabbits (Belloli *et al.*, 1991; Das *et al.*, 1992; Hoffsis *et al.*, 1990; Mondal *et al.*, 1994). The drugs included were streptomycin, isoniazid, clofazimine, rifampicin, ethambutol, pyrazinamide and dapsone as single drug therapy or in combination. The number of animals included in their study was small due to the cost of drugs. With a single drug treatment, the effect was either not noticeable or there was a transient clinical improvement with reduced fecal shedding. The improved condition lasted only for a few weeks and it was commonly followed by relapse while the animal was on treatment or immediately after the treatment ceased. In animals that were treated with a combination drugs such as streptomycin, isoniazid and rifampicin, the effect was more marked and cessation of *Map* being shed in the feces was prolonged. However, eradication was never achieved and persistent infection and relapse continued in the majority of these studies.

To control the spread of paratuberculosis, it is crucial to identify and cull infected animals in conjunction with an on-farm biosecurity program to develop and maintain a *Map*-free herd (NAHMS, 1997; Wells and Wagner, 2000). Control of paratuberculosis requires education of farmers with conviction and persistence to maintain the standards (Collins and Manning, 2002). However, farmers are unlikely to implement the measures needed to reduce the spread of the infection or comply with them unless *Map* infection has been identified in their herd. The long delay between infection and the development of clinical symptoms (Chiodini *et al.*, 1984; Manning and

Collins, 2001) may result in cows being culled from the herd before the disease is detected, leaving the farmer unaware of the presence of *Map* in the herd.

2.5 Vaccination

Immunization against *Map* was performed by subcutaneous injection of live attenuated bacilli between 1 to 30 days of age. Revaccination was not recommended. Many different variations of the vaccine have been evaluated, including attenuated; heat killed unattenuated strains; as well as disrupted fragments of *Map*. The use of live vaccine is not allowed in several countries such as The Netherlands due to the possibility of the attenuated strain entering the human food chain and the controversial debate on the link of *Map* to Crohn's disease in humans. Vaccination will reduce the number of animals with clinical disease and decrease the number of *Map* shedders and thus reduce the spread of the disease (Wentink *et al.*, 1994).

An important disadvantage to vaccination is the positive response of vaccinated animals to serodiagnostic tests for paratuberculosis (Kormendy, 1994; Spangler *et al.*, 1991). In vaccinated animals, diagnosis depends on fecal culture and PCR if the vaccination was administered as an inactivated strain. Vaccination also causes sensitization of the animal to bovine PPD used in the intradermal test to control and eradicate bovine tuberculosis (Stuart, 1965). Another undesirable feature of vaccination is the development of an inflammatory swelling and a subsequent nodule at the site of the inoculation. In several countries, vaccination has been used in order to control the disease. The adoption of a vaccination program should be considered in heavily infected

herds in which the hygiene practice and improved calf-rearing hygiene are not sufficient to control the disease. Vaccination will reduce losses to the farmer and will aid in economic survival.

The prevalence of paratuberculosis in the Alberta cattle population detected by serological technique is estimated to be 7%. The present algorithm for screening *Map* in Alberta is by a commercial ELISA kit from IDEEX on bovine fecal samples and positive ELISA samples are followed by culture technique. Because of the lack of sensitivity of both ELISA and fecal culture (Whitlock *et al.*, 1999), the incidence of disease may be under-reported. Johne's disease usually does not occur as an outbreak in which many animals are sick and die within a short period of time. For every infected animal showing clinical signs, 20 to 25 infected, asymptomatic animals may be present. The present method of infection control is by culling infected animals once they are identified. However, by this point in time, the majority of the animals in the herd may be infected or the infection may have been passed to the other herds especially if cross-pasturing is practiced. Molecular testing such as by PCR can improve the turn-around time for reporting; however, fecal specimens contain non-specific factors that coextract with DNA can interfere with the assay (Al-Soud and Radstrom, 2000; Wilde *et al.*, 1990). To circumvent this problem, this research program was undertaken to develop an immunomagnetic bead capture assay followed by amplification test for the detection of *Map* in fecal specimens. Development of such an assay will improve the sensitivity of detection and decrease the time required to make a diagnosis from several weeks to a

few days. This rapid testing will greatly improve control strategies for the detection and control of Johne's disease in cattle.

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3. Evaluation of four DNA extraction methods for the detection of *Mycobacterium avium* subsp. *paratuberculosis* by polymerase chain reaction

3.1 Introduction

Polymerase Chain reaction (PCR) is an amplification technique first described by Saiki *et al.* (1985). It is extremely useful because it facilitates the detection of fastidious or non culturable organisms with a rapid turn-around time. In Chapter 2, the fastidious nature of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) was described; therefore an amplification test using a specific target would be of great benefit in the diagnosis of Johne's disease. The target sequence chosen for the amplification in this study is the insertion element (IS*900*). It was first detected from a *Map* strain isolated from a Crohn's patient (Green *et al.*, 1989). The size of this repetitive DNA sequence is around 1.45 Kb, present in 18 to 20 copies in the genome of *Map* (McFadden *et al.*, 1987) and thus increases the sensitivity of the amplification assay compared with a gene that is found in single copy. IS*900* has been found in all reference and vaccine strains of *Map* and has also been used as a hybridization probe for the molecular characterization of *Map* isolates from different hosts (Bauerfeind *et al.*, 1996). Amplification of insertion element IS*900* has proven to be highly sensitive and specific for detecting *Map* strains (Collins *et al.*, 1989; Green *et al.*, 1989; Hermon-Taylor *et al.*, 2000).

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The success of amplification depends on the recovery of the target nucleic acid from clinical specimens such as blood, urine, feces and other tissues. However, there are nonspecific factors that coextract with target DNA or RNA causing inhibition in the PCR assays and thus result in amplification failure. Because the target of this study is to develop an assay for detecting *Map* in bovine feces, it is essential to select an appropriate DNA extraction method with high recovery of DNA. Therefore, the objective of this part of the study was to compare the sensitivity of PCR for the detection of *Map* employing four different DNA extraction methods in the presence or absence of bovine feces and also to develop a PCR internal control to be incorporated into each PCR tube to monitor the amplification and detect the presence of inhibitors.

3.2 Materials and methods

3.2.1 *Preparation of cell suspension*

Map ATCC (American Type Culture Collection, Manassas, VA) strain 19698 was grown in Middlebrook 7H9 broth at pH 7.0 (Becton Dickinsion Microbiology Systems, Sparkes, Maryland), supplemented with 2% Middlebrook Albumin Dextrose Catalase (Becton Dickinsion Microbiology Systems, Sparkes, Maryland), 0.5% Tween 80 (Sigma Chemical Co., St. Louis, Missouri), 0.04% Mycobactin J (Allied Monitor, Fayette, Missouri) and incubated at 35°C for 2 to 3 weeks. Cells were harvested by centrifugation at 1500 x *g* for 15 min. The pellet was washed twice with 5 ml of

phosphate-buffered saline, pH 7.2 (PBS) and adjusted to a concentration of 2 x 10^4–10^5 cells/ml based on the value of 1 OD_{550nm} = 2 x 10^6 to 10^7 cells/ml (Qiagen News, Issue #1, 1996; Qiagen, Mississauga, ON).

3.2.2 *Preparation of fecal spiked cell suspension*

Bovine feces samples that had previously tested negative for *Map* by the culture method were provided by Agri-Food Laboratories Branch, Alberta Agriculture, Food and Rural Development, Edmonton. All of these samples were stored at –70°C before use in spiking experiments. Five of these samples were thawed, pooled and thoroughly mixed to provide a homogeneous sample matrix to use for the following spiking experiments. One gram of feces was mixed with 1 ml of cell suspension (2 x 10^4 to 10^5 cells/ml) prepared above and diluted to a final volume of 5 ml with PBS, pH 7.2 (fecal spiked cell suspension). This mixture was extracted by different methods described below.

3.2.3 *DNA extraction*

Fifty microliters of the prepared cell suspension containing approximately 2 x 10^4 to 10^5 cells/ml were added to 200 µl of PBS, pH 7.2 (referred as the working cell suspension) and this concentration of cells was used for all of the experiments in this chapter. DNA was extracted from a 250 µl sample of the fecal spiked cell suspension performed in parallel with 50 µl of the undiluted cell suspension using the following methods:

1) Rapid extraction: 250 µl of lysis buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.3; 1 mM EDTA, pH 9.0; 1% Triton X-100) was added to an equal volume of working cell suspension or fecal spiked cell suspension. Both suspensions were boiled for 15 min and allowed to cool to room temperature. The disrupted cells were centrifuged at 13,000 x *g* for 15 min; the supernatant was removed and divided into 5 aliquots. Ten microliters of 3.0 M sodium acetate, pH 5.2 was added to 100 µl of supernatant followed by 1 ml of isopropanol before storing at -20°C for 2 h to precipitate the DNA. After washing with 70% ethanol, the DNA pellet was dried and re-dissolved in 10 µl of 12 mM Tris, pH 7.4. Five tubes of dissolved DNA were added to a single tube making a final volume of 50 µl.

2) Organic extraction: 250 µl of working cell suspension or fecal spiked cell suspension was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The suspension was mixed using a vortex for 30 s at 5 min intervals over a period of 30 min at room temperature. Following centrifugation at 13,000 x *g* for 10 min, the aqueous layer was removed and DNA was precipitated, washed, dried as described above and reconstituted in 50 µl of 12 mM Tris, pH 7.4.

3) Silica-based extraction: DNA was extracted according to the published method of Boom *et al.* (1990). Lysis buffer was prepared by dissolving 120 g of guanidine thiocyanate (Fluka Chemie AAG, Buchs, Switzerland) in 100 ml of 0.1 M Tris-HCl, pH 6.4 followed by addition of 22.0 ml of 0.2 M EDTA, pH 8.0 and 2.6 g of Triton X-100. One ml of the lysis buffer was added to 250 µl of working cell suspension or 250 µl of

fecal spiked cell suspension, followed by 40 µl of acid-washed silica. The suspension was mixed on a vortex for 10 s and held at 23°C for 15 min with constant mixing on a back to front rotator. The silica was pelleted by centrifugation at 13,000 x *g* for 30 s. The supernatant was discarded and the pellet was washed twice with buffer containing 12% guanidine thiocyanate in 0.1 M Tris-HCl, pH 6.4 followed by 2 washes with 70% ethanol and one with acetone. The pellet was dried at 56°C for 5 min and then reconstituted in 50 µl of 12 mM Tris-HCl, pH 7.4.

4) MagaZorb® DNA isolation: DNA was extracted according to the manufacturer's instructions using the reagents provided with the MagaZorb® DNA isolation kit (Cortex Biochem, San Leandro, CA). Briefly, 250 µl of working cell suspension or fecal spiked cell suspension was added to 20 µl of PK solution followed by the addition of 200 µl of Lysis Buffer. After vortexing for 15 s, the tubes were held for 10 min at 56°C. Five hundred microliters of Binding Buffer and 20 µl of magnetic particles were added and mixed at room temperature for 10 min using a front to back rotator. Dynal MPC®-S (Dynal magnetic particle concentrator, Dynal Biotech LLC, Brown Deer, WI), a rack with a removal magnetic backing was used to sediment the particle-bound DNA. After 3 washings with 1 ml of Wash Buffer, the MagaZorb® particles were sedimented using the magnetic rack. DNA was eluted from the particles by adding 50 µl of 12 mM Tris-HCl, pH 7.4 and mixing for 10 min at 56°C before using Dynal MPC®-S to separate the particles from the DNA solution.

DNA extracted from the above procedures was diluted from 10^{-1} to 10^{-7} with 12 mM Tris, pH 7.4 for use as templates in the PCR titration assays. Each of the four extraction methods was repeated a total of 4 times using the same pool of fecal spiked cell suspension which was stored at 4°C between runs.

3.2.4 *Determination of sensitivity*

A cell suspension containing 2 x 10^4 to 10^5 cells/ml was diluted from 10^{-1} to 10^{-4}. One milliliter of each dilution was added to 1 *g* of bovine feces and thoroughly mixed. PBS, pH 7.2 was added to a final volume of 5 ml and the suspensions were mixed again. DNA from a 250 µl aliquot of each of the fecal spiked cell suspensions was extracted using the MagaZorb® DNA isolation kit and it was eluted in a final volume of 50 µl. Likewise, 50 µl aliquots of the 10^{-1} to 10^{-4} dilutions of the above cell suspension were extracted as described above to compare the efficiency of the extraction procedure in undiluted cell versus fecal spiked cell suspensions.

3.2.5 *Construction of an internal control (IC) and PCR*

An internal control was constructed for the detection of inhibition in PCR. Fifty microliters of *Escherichia coli* DH5α competent cells were transformed with 50 ng of plasmid 54286 pcDNA3.1(+) DNA (Invitrogen Canada Inc., Burlington, ON) and plated onto Luria-Bertani (LB) plates containing 50 µg of ampicillin /ml. Single colonies selected from the LB agar plates were grown in LB broth containing 50 µg of ampicillin /ml and the plasmid was isolated using the Rapid Plasmid Miniprep Kit (Invitrogen)

according to the manufacturer's instructions. Using the plasmid sequence provided in the kit, a set of primers was designed with the J5A and J5B primers as 5' overhangs to produce a 992 base pair product as an internal control (IC) for *Map* PCR. The primer sequences are as follows: bases in italics representing the J5A and J5B *Map* primer overhangs, respectively:

Forward 5'-*ATGTGGTTGCTGTGTTGGATGG*CCCGGGAGCTTGTAT-3'

Reverse 5'-*CCGCCGCAATCAACTCCAG*AGCCCAACCTTTCATA-3'.

The IC was generated using the following cycling parameters: 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 2 min with a final extension at 72°C for 5 min. The PCR product was analyzed on a 1% agarose gel and the IC band was excised and the DNA was eluted from the agarose using the QIAEX II gel extraction kit (Qiagen Inc., Mississauga, ON) according to the manufacturer's instructions. The IC product was used as a template with *Map* PCR primers to ensure that no other bands were formed. The amount of IC to be added to each PCR reaction was optimized by titration with *Map* DNA to ensure that it did not interfere with amplification of the target *Map* DNA. This determined the quantity that would be used as IC for all conventional PCR assays performed in this study.

Conventional PCR was performed using a ABI 9600 (Applied Biosystem Inc., CA) with established primers (Hermon–Taylor *et al.*, 2000) designated J5A (Forward 5'-*ATGTGGTTGCTGTGTTGGATGG*-3', and J5B (Reverse 5'-

CCGCCGCAATCAACTCCAG-3') targeting the insertion element IS*900* of *Map*. The size of the amplified product is 298 bp. Ten µl of extracted DNA was added to a cocktail containing 1 x PCR buffer (Invitrogen), 1.5 mM magnesium chloride (Invitrogen), 0.1 mM dNTP (Invitrogen), 0.75 µM of each primer and 1 U Platinum™ Taq polymerase (Invitrogen). One microliter of 300 fg/ml of internal control DNA was added to each reaction tube. Cycling parameters were as follows: 94°C for 5 min; [94°C for 30 s, 66°C for 30 s and 72°C for 1 min] x 4 cycles; [94°C for 30 s, 64°C for 30 s and 72°C for 1 min] x 4 cycles; [94°C for 30 s, 62°C for 30 s and 72°C for 1 min] x 4 cycles, [94°C for 30 s, 58°C for 30 s and 72°C for 1 min] x 24 cycles and a final extension at 72°C for 7 min. The amplicons were detected by electrophoresis in 1% agarose gels containing 0.5% ethidium bromide and visualization by UV illumination. A kb DNA size marker was included in each gel (Invitrogen).

3.3 Results

3.3.1 *Internal control*

The primers selected from IS*900* demonstrated no cross-reactivity with the plasmid chosen for the sequence design of the internal control. PCR using J5A and J5B primers directed to the insertion element IS*900* detected 0.3 femtograms of IC template. In the presence of 3 fg of IC, 10 fg of *Map* template can be detected using J5A and J5B primers (Fig 3.1).

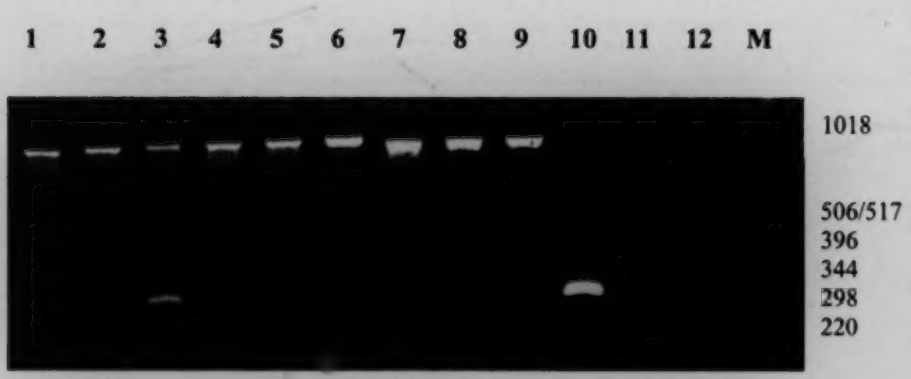

Figure 3.1. PCR results with different concentrations of IC and *Map* DNA. 10 fg of *Map* DNA was coamplified with 30 fg, 3 fg and 0.3 fg of IC in lanes 1 to 3. 1 fg of *Map* DNA was coamplified with 30 fg, 3 fg and 0.3 fg of IC in lanes 4 to 6. 30 fg, 3 fg and 0.3 fg of IC was used in the PCR assay in lanes 7 to 9. Lane 10: 10 fg of *Map* DNA. Lane 11: 1 fg of *Map* DNA. Lane 12: negative water control. M: 1kb molecular weight marker in base pairs.

3.3.2 *Comparison of extraction methods for cell and fecal spiked cell suspensions*

A summary of the PCR endpoint titration for extracted DNA from the working cell and fecal spiked cell suspensions is shown in Table 3.1. The PCR endpoint was a dilution of 10^{-4} when DNA was extracted using the rapid lysis method and organic extraction methods. DNA extractions by silica-based technology and the MagaZorb® DNA isolation kit had identical PCR endpoint titrations of 10^{-5} dilution. Extractions using the fecal spiked cell suspension exhibited inhibition with all four extraction methods. The incorporation of the internal control with target DNA in the same amplification reaction tube enabled the detection of inhibition. A 1/1000 dilution of the extracted DNA was required to eliminate the inhibitory factors using the rapid lysis, organic and silica-based extraction methods for the fecal spiked cell suspension. Using the MagaZorb® kit, a 1/100 dilution of the extracted DNA was needed to remove the effect of the inhibitors. There was no difference in the results of the PCR endpoint titrations between working cell and bovine feces spiked with *Map* cells by each of the different extraction methods (Table 3.1).

Table 3.1. Summary of PCR endpoint titration with working cell[1] and fecal spiked cell[2] suspensions.

DNA	Rapid Lysis		Organic		Silica-based		MagaZorb®	
	Cell	Fecal	Cell	Fecal	Cell	Fecal	Cell	Fecal
undiluted	+	I	+	I	+	I	+	I
10^{-1}	+	I	+	I	+	I	+	I
10^{-2}	+	I	+	I	+	+	+	+
10^{-3}	+	+	+	+	+	I	+	+
10^{-4}	+	+	+	+	+	+	+	+
10^{-5}	-	-	-	-	W+	W+	+	+
10^{-6}	-	-	-	-	-	-	-	-
10^{-7}	-	-	-	-	-	-	-	-

Results of 4 replicates using same pooled feces.

+ : PCR positive; W+: weak positive (faint band on gel); - : PCR negative; I: inhibition. Working cell suspension[1] was prepared by adding 50 µl of approximately 2 X 10^4 to10^5 cells/ml to 200 µl of PBS, pH 7.2 and DNA was extracted from this volume of cells. Fecal spiked cell[2] suspension was prepared by adding 2 x 10^4-10^5 cells to 1 gram of bovine feces and diluted to a final volume of 5 ml and 250 µl was used for DNA extraction.

3.3.3 *Comparison of sensitivity*

The sensitivities of PCR using DNA extracted by the MagaZorb® isolation kit from the 10^{-1} to 10^{-4} of dilutions of cells (Figure 3.2) and bovine feces spiked with cell suspensions (Figure 3.3) were compared. Based on the starting concentration, the amount of extracted DNA from *Map* cells used for the PCR assay extracted in lanes 1 to 4 of Figure 3.2 were 100 to 1000, 10 to 100, 1.0 to 10 cells and 1 cell or less, respectively. Results using the same number of cells in the presence of fecal material are represented by lanes 1 (100 to 1000), 5 (10 to 100), 9 (1.0 to 10 cells) and 13 (1 or less) in Figure 3.3. No PCR product was observed in any of these lanes due to the presence of inhibitors that prevented amplification. Upon 1:100 dilution of the DNA extracted from bovine feces spiked with cells, PCR product was seen in the 100 to 1000 (lanes 3 and 4) and 10 to 100 (lane 7) cells but not in samples of 1 to 10 cells (lanes 10 to 12) or less than 1 cell (lanes 15 to16). Thus, there was a loss of one log of sensitivity in the presence of bovine feces and amplification only occurred after dilution of the extracted DNA.

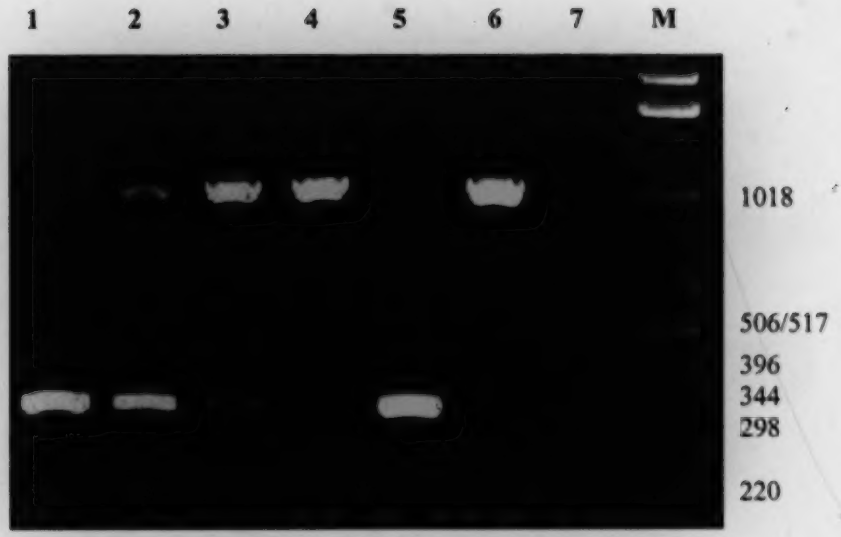

Figure 3.2. Sensitivity of PCR amplification of DNA extracted from different concentrations of *Map* cells by MagaZorb®, a DNA extraction kit. Lane 1 : 100 to 1000 cells. Lane 2: 10 to 100 cells. Lane 3: 1 to 10 cells. Lane 4: 1 cell or less. Lane 5: Internal control and positive control *Map* DNA. Lane 6: Internal control DNA. Lane 7: Negative control. Molecular weight marker (M): 1 kb ladder. The size of the PCR product is 298 bp and the size of the amplified internal control is 992 bp.

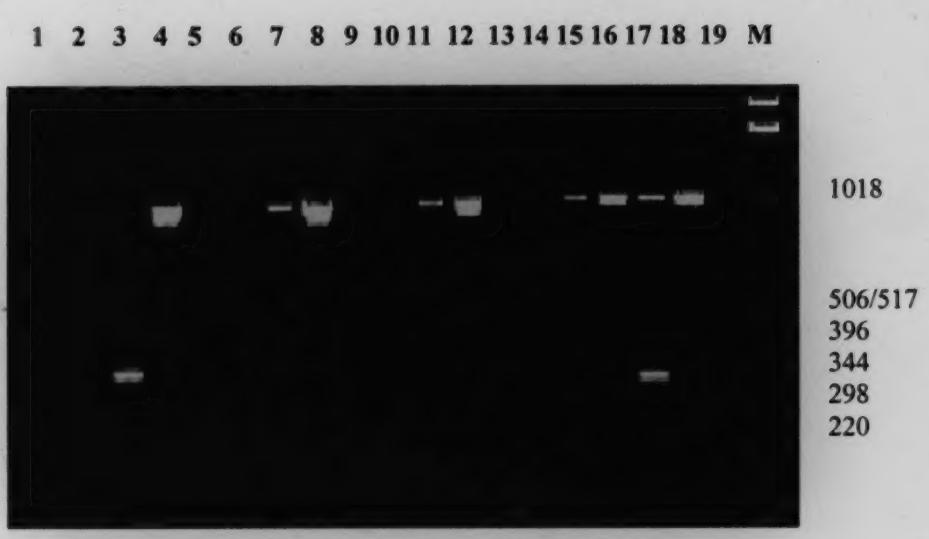

Figure 3.3. Sensitivity of PCR amplification of DNA extracted by MagaZorb® from bovine feces spiked with different concentrations of cells. Lanes 1-4: DNA from 100 to 1000 cells; undiluted and dilutions from 10^{-1}-10^{-3}. Lanes 5-8: DNA from 10 to 100 cells; undiluted and dilutions from 10^{-1}-10^{-3}. Lanes 9-12: DNA from 1 to 10 cells; undiluted and dilutions from 10^{-1}-10^{-3}. Lanes 13-16: DNA from less than 1.0 cell; undiluted and dilutions from 10^{-1}-10^{-3}. Lane 17: Internal control and positive control *Map* DNA. Lane 18: Internal control. Lane 19: Negative control. Molecular weight marker (M): 1kb ladder

The extraction methods were also compared for the ease of extraction, the amount of labor required and completion time (Table 3.2). All four extraction methods are easy to perform but the hands-on time is different. Magnetic-based technology (MagaZorb® DNA isolation kit) has the shortest time of completion. Because labor is an expensive component of many tests, consideration has to be given to choosing the simplest protocol with the least labor and shortest completion time.

Table 3.2 Comparison of four different methods for DNA extraction and the following results were based on five samples/extraction in a run.

Method	Labor (min)	Time for completion (min)
Rapid lysis	34	220
Organic	44	224
Silica-based	41	67
MagaZorb®	19	50

3.4 Discussion

Polymerase chain reaction is a powerful tool for detecting target nucleic acids from pathogens in clinical specimens. Unfortunately, its application in feces has been limited because of the presence of inhibitory substances such as bile salts, bilirubin, urobilinogens, polysaccharides and heavy metals (Al-Soud and Radstrom, 2000; Wilde *et al.*, 1990). Conventional methods of extraction such as organic solvents (phenol-chloroform-isoamyl alcohol) failed to remove inhibitors even with multiple treatments. In order to improve the recovery of the target nucleic acid to circumvent the problem of inhibition, other extraction methods have been developed such as column chromatography with Sephadex G25, G50, Sepharose CL-6B (Lou *et al.*, 1997), silica powder (Brian *et al.*, 1992) or ion exchange chromotography after phenol-chloroform extraction (Kato *et al.*, 1993). Chaotrophic agents (Caeiro *et al.*, 1999; Stacy-Phipps *et al.*, 1995) and various binding media such as cellulose fiber powder (Wilde *et al.*, 1990), polyethylene glycol 6000 (De Wit *et al.*, 1990), polyvinyl pyrrolidine (Lawson *et al.*, 1998), glass matrix have been employed. Commercial extraction kits (Gumerlock *et al.*, 1993; Ibrahim *et al.*, 1992) and combinations of rapid extraction methods with commercial kits (Fahle and Fischer, 2000; Holland *et al.*, 2000) have also been used to isolate DNA from different types of samples. An alternative way to eliminate the effect of inhibitors in PCR is to dilute the extracted nucleic acid; however, the sensitivity of the assay is compromised (Allard *et al.*, 1990; Wilde *et al.*, 1990; Stabel *et al.*, 2004) and this was also observed in this study. There was a ten fold drop in sensitivity of the test

when fecal spiked cells were compared with PCR endpoint titration on cell suspension without bovine feces.

Improvements in sensitivity have been reported with nested PCR (Hornes *et al.*, 1991), booster PCR (Saulnier and Andremont, 1992) or reamplification of PCR products (Pierre *et al.*, 1991). Wiedbrauk *et al.* (1995) showed that DNA polymerases isolated from *Thermus thermophilus* and *Thermus flavus* are less sensitive to inhibitory substances.

Culture enrichment can circumvent the problem of inhibitors in feces by diluting out the inhibitors with additional culture medium and by increasing the number of target organisms through growth before extraction and amplification (Holland *et al.*, 2000). However, *Map* is such a slow growing organism so this is not a practical approach. The complex nature of bovine feces creates the need to investigate the possibility of using different methods to overcome the difficulties in obtaining target nucleic acid for amplification. Monteiro *et al.* (1997) identified a complex polysaccharide found in human feces that is a PCR inhibitor that possibly originates from dietary vegetable material.

In this study, an internal control was constructed using the same set of PCR primers as the target. Co-amplification of an internal control by the *Map* primers in each PCR reaction allowed the detection of inhibitors in each reaction tube. Failure to generate amplicons from the internal control can also be indicative of failure in the PCR assay itself due to errors in reagent preparation or incorrect cycling parameters. The

amplicon generated from the internal control was designed to be much larger than the target amplicon (992 vs 298 bp), allowing the preferential amplification of the target DNA. Too high a concentration of the target DNA will compete with the internal control for primers resulting in a negative or weak internal control result (Figure 3.2, lane 1 and 5). In contrast, a non-competitive internal control would test for the presence of inhibitors; but does not monitor the performance of the target-specific primers. Internal controls are of great importance because they help to detect PCR failure and thus reduce the possibility of detecting false negative results and was clearly demonstrated.

In this study, DNA was extracted from approximately 1000 to 10,000 cells using rapid lysis, organic and silica-based technologies and the MagaZorb® DNA isolation kit. By making 10-fold serial dilutions of the DNA before amplification, the endpoints of detection were identical (10^{-5} dilution) for silica-based and magnetic-based technologies indicating that the efficiency of extraction is similar for these methods. The endpoint PCR for rapid lysis and organic extraction was one log lower than the other two methods. In the presence of bovine feces, the PCR endpoint titration of each of the extraction methods was the same as in the absence of fecal material (Table 3.1); however, dilution of the DNA was required to circumvent the effect of inhibitors. The magnetic-based MagaZorb® DNA isolation kit was the most efficient because only a 1/100 dilution is neccessary to remove the inhibitory effect; however, further testing using different sources of bovine feces is necessary to confirm if this observation is

reliable. The need for dilution is detrimental to the testing procedure especially when the bacterial load in the test sample is low and inhibitors are present.

With feces, the sensitivity of extraction by MagaZorb® DNA isolation kit using endpoint PCR titration is at the level of 2 to 20 cells as calculated from the amount of DNA extracted from 10 to 100 cells adjusted by the amount of DNA that was used for the PCR assay (Figure 3.3). Applying the same calculation when the working cell suspension was used in the extraction indicates that the sensitivity of detection is less than 2 cells in the absence of feces (Figure 3.2).

The labor required to extract DNA differs from one method to the other. The completion time for extraction using rapid lysis and organic extraction was similar but these two methods took longer than the silica-based method and the MagaZorb® DNA isolation kit. Generally, labor cost is the most expensive component of an extraction procedure; therefore, consideration should be given to the protocol that requires the least "hands-on" time. The MagaZorb® DNA isolation kit is more expensive than the cost of reagents and reagent preparation for the other three assays. However, with the shortest "hands-on" time, this kit is the most efficient and cost effective way of performing DNA extraction for *Map* PCR.

3.4 References

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4. Immunological response to *Mycobacterium avium* subsp. *paratuberculosis* in chickens. *

4.1 Introduction

In the previous chapter, the purification of DNA using magnetic bead extraction was shown to be superior and less labor intensive compared with other methods included in the study. However, fecal spiked cells contained inhibitory factors that coextracted with the target DNA and dilution was required to circumvent the problem of PCR inhibition. For specimens with a low bacterial load, the dilution step would result in the loss of the target. Consequently an alternative method was explored using a polyclonal antibody to capture *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) from bovine feces before DNA extraction. This allows specific capture and concentration of the target organism and reduces the chance of co-extracting either non-specific DNA or inhibitory factors found in feces.

Antibodies have been widely used in research and diagnostic applications and rabbits are frequently used for production of specific polyclonal antibodies. In recent years, focus on production of polyclonal antibodies has shifted from mammals to avian species. Chickens are commonly used to produce polyclonal antibodies to conserved mammalian proteins because of their evolutionary distance from mammals (Gassmann *et al.*, 1990). Chicken eggs have been used as an excellent source of polyclonal antibodies in many studies (Chang *et al.*, 1999; Hatta *et al.*, 1993; Sunwoo *et al.*, 2002; Yokoyama *et al.*, 1998a, b).

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Each bird can produce five to six eggs per week with a yolk volume of approximately 5 to 8 ml per egg. Furthermore, the process of bleeding rabbits is invasive and far more stressful to the animal compared with collecting eggs from a chicken. The primary shortcoming of using egg-derived antibodies is that the extraction of immunoglobulin from the yolk is more labor intensive and time consuming than the preparation of immunoglobulin from mammalian sera.

There are three major immunoglobulin classes in chickens: IgG (referred to as IgY), IgA and IgM (Schranner and Losch, 1986). During the maturation of the egg in the oviduct, active transport of IgY from the chicken's serum to the yolk results in significant IgY levels in the yolk (Rose *et al.*, 1974). IgY does not bind to protein A (Kronvall *et al.*, 1970) or protein G (Larsson *et al.*, 1993), consequently, the purification of IgY differs from the purification of mammalian IgG.

The production of IgY is simple and has many applications. Therefore, the goal was to immunize chickens with *Map* for large-scale production of antibody and to evaluate its specificity and sensitivity in capturing this organism for future development of an immunocapture-PCR for the detection of *Map*.

4.2 Materials and methods

4.2.1 *Bacterial strains and cultures*

Field strains of *Map*, FR2616, AA3814, EA4146, EQ2356, ER2945Y162, 11992, 12258, 4200 and 11520-5 were obtained from the Agri-Food Laboratories Branch, Alberta Agriculture, Food and Rural Development, Edmonton, Alberta. These field isolates were confirmed to be *Map* by culture technique and biochemical testing performed at the Mycobacteria Division of the Provincial Laboratory for Public Health (Microbiology). In addition, the following strains were included in the study to test for specificity of the polycloncal antibody produced: *Mycobacterium avium* (ATCC 25291), *M. gordonae* (ATCC 14470), *M. intracellulare* (ATCC 13950), BCG (Bacillus Calmette Guérin), a bovine attenuated mutant cultured several hundred times on bile containing culture media used as a vaccination strain for humans against tuberculosis. *M. tuberculosis* H37Ra (ATCC 2177) and two control isolates of *Map* ATCC 19698 and ATCC 43544 purchased from the American Type Culture Collection (Manassas, VA).

4.2.2 *DNA extraction, restriction digestion and Southern hybridization*

Cultures of *Map* ATCC 19698 and 43544, and the *Map* field strains, EQ2356, FR2616, EA4146 and AA3814 were grown in Middlebrook 7H9 liquid medium at 36°C for 8 weeks. The cells were harvested by centrifugation at 2500 x g for 20 min and the pellet was washed twice in phosphate-buffered saline (PBS), pH 7.4. Concentration of the cell suspension was standardized to 2 X 10^4 to 10^5 cells/ml and 1 ml of cells was pelleted and used for DNA extraction. Two hundred microliters of siliconized GLC 40

mesh glass beads (BDH, Toronto, ON) was added to the pellet and vigorously mixed by vortex to resuspend the pellet. After adding 500 µl of TE buffer (Tris-EDTA, pH 8.4), an equal volume of phenol-chloroform was added. The mixture was mixed by vortex for 1 min, followed by centrifugation at 13,500 x *g* for 10 min. The aqueous layer was removed to another microcentrifuge tube and the phenol-chloroform extraction step was repeated. After centrifugation at 13,500 x *g*, the aqueous layer was removed and it was extracted with chloroform. After a final centrifugation at 13,500 x *g*, the aqueous layer was removed and 1:10 volume of 3.0 M sodium acetate, pH 5.2 was added followed by 5 volumes of isopropanol to precipitate the DNA. The concentration of DNA was quantified using a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech Inc., San Francisco, CA). One-hundred and fifty nanograms of DNA was digested using 1 unit of *Bst*EII (Invitrogen, Burlington, ON) or *Pvu*II (Invitrogen) at 37°C for 3 h. The digested DNA was subjected to electrophoresis in a 0.8% agarose gel at 35V for 22 h. The DNA was denatured in 0.5 M NaOH / 1.5 M NaCl for 15 min and neutralized in 1 M Tris / 1.5 M NaCl buffer (pH 8.0) for 1 h. The DNA fragments were passively transferred from the gel to a Hybond N nylon membrane (Amersham Corporation, Arlington Heights, IL.) by a standard blotting protocol (Sambrook *et al.*, 1989) using 6 x SSC (0.5 M sodium chloride, 0.015 M sodium citrate, pH 7.0) buffer (0.9 M NaCl / 0.09 M Na_3-Citrate-$2H_2O$). DNA fragments were cross-linked to the membrane using the cross-linking program preset for "Southern damp membrane" in the GS Gene Linker UV chamber (BioRad, Mississauga, ON). The membrane was stored at -20°C until required for Southern hybridization.

The DIG (digoxigenin) probe for Southern hybridization was prepared using PCR and established primers designated J5A (Forward 5'-*ATGTGGTTGCTGTGTTGGATGG*-3'), and J5B (Reverse 5'-*CCGCCGCAATCAACTCCAG*-3) (Hermon-Taylor *et al.*, 2000) targeting the *Map* IS*900* insertion element with an amplicon size of 298 bp. Ten femtograms of the extracted DNA was added to a solution containing 1 x PCR buffer, 1.5 mM magnesium chloride, 0.1 mM dNTPs of DIG (digoxigenin) DNA labelling mix (Roche-Boehringer Mannheim, Mississauga, ON) and 0.75 µM of each primer. The conditions for the PCR were as described on section 3.2.5. The amplicons were detected by electrophoresis in a 1% agarose gel containing 0.5% ethidium bromide and visualed by UV illumination. A 1 kb DNA size marker was included in each gel (Invitrogen). The amplicons were purified using the QIAquick PCR purification Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The amount of DIG labelling in the purified amplicon was determined by visual comparison of color to a set of DNA standards dotted on a control strip (DIG Quantification Teststrips, Roche-Boehringer, Mannheim). One microliter of the DIG-labelled purified amplicon was applied to the test strip provided by the kit and dilutions of the standards of DIG-labelled DNA (300 pg/ml, 100 pg/ml, 10 pg/ml, 3 pg/ml) and DIG-labelled control DNA included in the kit was also applied on the same strip. After 5 min holding at 23ºC, the test strip was submerged into solutions supplied by the manufacturer for the following durations: blocking solution: 5 min; antibody binding solution: 3 min; blocking solution: 1 min; washing solution: 1 min; equibration solution: 1 min; color reaction: 5 to 30 min holding in the dark depending on the

development of color. Visual examination was performed and the color of the test sample was compared to the color of the standards.

Southern hybridization was performed using the standardized protocol previously established for restricted fragment length polymorphism in generating fingerprint profiles for *M. tuberculosis* in the Provincial Laboratory for Public Health Laboratory (Microbiology) in Edmonton, Alberta. The membrane was prehybridized at 68°C for 2 h with rocking in 20 ml of pre-hybridization solution consisting of 6 x SSC (0.5 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 5 x Denhardt solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin, 1.0% sodium dodecyl sulfate) and 100 µg of denatured salmon sperm DNA (Sigma-Aldrich Canada Ltd., Oakville, ON) per ml. After prehybridization, the denatured DIG labelled hybridization probe prepared as describe above was added to give a final concentration of 15 pg/20 ml of hybridizing buffer. Hybridization was done at 68°C overnight in a circulating water bath. Membranes were rinsed twice in 6 x SSC (pH 7.0) and once in 6 x SSC with 0.1% SDS at 68°C for 1.5 h. The membranes were rinsed briefly with 1 x maleate buffer (0.1 M maleaic acid / 0.15M sodium chloride) followed by 1 x maleate buffer with 1.5% blocking solution (Roche Diagnostics Corporation, Indianapolis, IN) for at room temperature for 2 h. The buffer was replaced with 1 x maleate buffer containing 3 units of anti-digoxigenin, 1% blocking reagent and held at room temperature for 1 h. After four washes of 15 min each with 1 x maleate buffer with 0.3% Tween 20, the membrane was equilibrated with Buffer C (100 mM Tris / 100 mM NaCl) for 5 min before incubating the membrane in 0.25 mM of CSPD (disodium 3-(4-methoxyspiro{1,2-

dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate) [Roche Diagnostics Corporation, Indianapolis, IN] in buffer C containing 0.1% magnesium chloride at 37°C for 5 min. The membrane was placed in X ray cassettes with X-Omat AR X-ray film (Eastman Co., Rochester, NY) and developed. The bands were examined visually and analysis was done with the Bio-Rad Gel Doc System and BioNumerics Software (Applied Maths Inc. Austin, TX). A dendrogram was generated using Dice coefficient with 1% tolerance (Tyrrell *et al.*, 2002).

4.2.3 *Immunization of chickens*

Single Comb White Leghorn (SCWL) chickens at age 23 weeks were housed and cared for in accordance with the Canadian Council on Animal Care guidelines for animal welfare. They were immunized according to the procedure described by Sunwoo *et al.*, (1996). Four different strains of *Map* (FR2616, AA3814, EA4146 and EQ2356) were harvested from Herrold Egg Yolk Medium supplemented with mycobactin (HEYM) slants after 4 to 6 weeks post inoculation. The cells were suspended in sterile phosphate-buffer-saline (PBS), pH 7.4 and centrifuged at 2500 x g for 20 min. The pellet was suspended in PBS and the cell density was adjusted to a McFarland # 1 standard and equal volume of cells from the above 4 different strains was added to 50 ml centrifuge tube to constitute the pool of *Map* cells. For inactivation, the cells were heated for 1 h at 90°C or suspended in 10% formalin-PBS held at room temperature for 24 h. After the inactivation steps, the formalin was removed by washing the cells three times with 50 ml of PBS, pH 7.4. Both sets of inactivated *Map* cells were dispensed into

3 ml aliquots and stored at 4°C. These two pools of heat- or formalin-inactivated cells were used for the immunization of chickens throughout the course of this study. The cells were tested for viability by inoculating 200 µl on HEYM slants in quadruplicate.

Cells were emulsified with an equal volume of Freund's incomplete adjuvant (Fisher Scientific, Nepean, ON) and injected intramuscularly into each side of the breast muscle of the chickens using 0.5 ml per site. Each prepared pool of *Map* cells was inoculated into two chickens. Booster immunizations were given 3 and 7 weeks after the initial immunization. Eggs were collected daily from week 1 to week 13 and stored at 4°C until processed. For the control chickens, PBS without *Map* was injected in an identical manner.

4.2.4 *Purification of IgY*

The IgY antibody was purified using the EggSTRACT®kit, (Promega Corporation, Madison, WI) according to the manufacturer's instruction. The egg separator and solutions used in this section of the study were provided with the kit. Briefly, each egg-yolk was separated from the white using an egg separator. The yolk membranes were ruptured and the volume of the yolk was measured. Three volumes of Precipitation Solution A were added to the yolk while stirring with a magnetic stir bar. The precipitated mixture was centrifuged at 10,000 x *g* for 10 min at 4°C. The supernatant was filtered through sterile gauze and the volume was measured. One part of Precipitation Solution B was slowly added to three parts of supernatant while stirring on a magnetic stirrer at room temperature to precipitate the IgY. After centrifugation at

10,000 x *g* for 10 min at 4°C, the supernatant was discarded and the pellet was dissolved in a volume of PBS equal to the original volume of the yolk as measured previously and stored at –20°C. The protein concentration of the purified IgY was determined using the Bio-Rad Protein Assay (Bio-Rad, Mississauga, ON). IgY preparations were examined for purity by comparison to an IgY standard (Promega) using polyacrylamide gel electrophoresis (PAGE). IgY samples were loaded on to a precast 12% polyacrylamide minigel (NuPAGE® Bis-Tris SDS-PAGE; Invitrogen) using an Xcell *Sure*Lock™ Mini-Cell (Invitrogen) and electrophoresis was run at 200V for 45 min. The bands were visualized by staining the gel with SimplyBlue™ SafeStain (Invitrogen) according to the manufacturer's instructions.

4.2.5 *ELISA, agglutination and immunofluorescent assays*

The immune response titer of the IgY to inactivated *Map* by heat or formalin was monitored by ELISA using the IDEXX Johne's test kit (IDEXX Laboratories, Westbrook, ME). The secondary antibody (anti-bovine IgG) was replaced with anti-chicken IgY (Southern Biotech Associates, Inc., Birmingham, AL). The testing procedure was done according to the IDEXX version of *Map* ELISA protocols (Herdchek *Map* Antibody Test Kit; IDEXX). Purified IgY samples diluted from 1:500 to 1:16,000 were added to the *Map* antigen-coated 96-well microtiter plate and held at 37°C for 30 min. After holding at 37°C, the wells were washed four times with 350 µl of PBS pH 7.4 (Invitrogen) in an automatic microtiter plate washer (Spectramax 340 PC, Molecular Devices, Sunnyvale, CA). A 100 µl volume of diluted rabbit anti-chicken

IgY:HRPO (horseradish peroxidase) conjugate (Promega Coporation) was added to each well and the plate was held at room temperature for 30 min. The washing step was repeated (as above), followed by the addition of 100 µl of TMB (3,3' 5,5' Tetramethybenzidine; IDEXX) substrate solution to develop the enzymatic color reaction. The reaction was stopped by adding 100 µl of 0.125% hydrofluoric acid (IDEXX). Optical density (OD) values were read at 660 nm using a Vmax microtiter plate reader (Spectramax 340 PC, Molecular Devices, Sunnyvale, CA). Initially, three randomly chosen eggs were selected each week to determine the immune response to *Map*. After an immune response was detected, all IgY preparations from subsequent weeks were tested.

Specificity of the IgY for immunocapture to *Map* and other closely related mycobacteria was determined using agglutination assay and immunofluorescent (IF) microscopy. The following strains were standardized to a concentration of 10^4 cells/ml: *Map* strains FR2616 and ATCC 19698, *M. avium, M. gordonae, M. intracellulare*, BCG, *M. tuberculosis* H37Ra. For the agglutination test, 20 µl of undiluted and 1:50, 1:100, 1:200, 1:500, 1:750 and 1:1000 dilutions of IgY was added to a clean slide and 5 µl of cell suspension was mixed with the IgY by tilting the slide several times and agglutination was checked. In the immunofluorescent protocol, 100 µl of standardized cells was pelleted and resuspended in 1 ml of IgY containing dilutions of 1:50, 1:100, 1:250, 1:500, 1:750. 1:1,000, 1:1,500 and 1:2,000 in phosphate buffer saline (PBS). After 1 h of incubation at 37°C, the cells were washed 5 x with PBS, pH 7.4. The cells were resuspended in 100 µl of PBS and goat anti-chicken IgG (H and L)- FITC

(Southern Biotech Associates, Inc.) at a final concentration of 1:20 as specified by the manufacturer. Following holding at 37°C for 30 min, the cells were washed as described above and resuspended in 100 µl of PBS. Ten microliters of cells was placed on glass slides and examined using an epifluoresecent microscope (Zeiss Axioskop 20). IgY preparations were subjected to a pre-adsorption protocol to eliminate cross reactivity with *M. avium*. Five milliliters of *M. avium* cells adjusted to a concentration of 10^8 cells/ml was centrifuged at 13,000 x *g* for 5 min and the pellet was resuspended in 5 ml of pooled IgY (titer > 1/2000). The cell suspension was held at 4°C, with rocking overnight, followed by centrifugation at 13,000 x *g* and the supernatant containing the IgY was removed and filtered through a 0.22 µm filter (Millipore Canada Ltd., Nepean, ON) and retested with *Map* and *M. avium* cells.

4.2.6 *Immunocapture assay and PCR*

To confirm the binding specificity of the different *Map* and other *Mycobacterium* strains, two hundred microliter volumes of each of the above bacterial cell suspensions was centrifuged at 13,000 x *g* for 5 min. The pellet was resuspended in 200 µl of pre-adsorbed antibody at a final concentration of 1/500 for 1 h followed by addition of 2 µl of MagaBeads™ rabbit anti-chicken IgG and hold at room temperature for 1 h. After 5 washes with PBS to remove unbounded bacteria, 1% SDS in 12 mM Tris-HCl (pH 7.4) was added to the MagaBeads™ complex and held at room temperature for 15 min. The reaction tube was placed in the MPC®-S unit (DyNal Biotech LLC. WI), the beads were separated from the supernatant and DNA was extracted from the supernatant using the

MagaZorb® DNA isolation kit (Cortex BioChem, San Leandro, CA). All the solutions used for the extraction were from the MagaZorb® DNA isolation kit. The elution buffer from the kit was not used because of the presence of EDTA. At the concentration found in the buffer, EDTA may interfere with the PCR and therefore DNA was eluted with 12 mM Tris buffer instead. Briefly, 200 µl of eluted bacterial cells was added to 20 µl of PK solution, followed by the addition of 200 µl of Lysis Buffer in a 1.5 microcentrifuge tube. After mixing by vortex for 15 s, the tubes were held at 56°C for 10 min. Five hundred microliters of Binding Buffer and 20 µl of magnetic particles were added, followed by mixing at room temperature for 10 min, the tube was placed in in the MPC®-S unit (DyNal Biotech LLC) to separate the particle-bound DNA from the solution. After three washings with 1 ml of Wash Buffer, the DNA was eluted from the beads by adding 200 µl of 12 mM Tris-HCl, (pH 7.4) followed by mixing at 56°C for 10 minutes. The particles were separated from the DNA solution by placing the tube in the MPC®-S (DyNal Biotech LLC).

To determine the specificity of the immunocapture, PCR assays were run using *Map* specific primers J5A and J5B and universal primers directed to the 23S rRNA sequence (Anthony *et al.,* 2000). The primer sequences for the latter target were as follows: Forward-5' *GCGATTTCYGAAYGGGGRAACCC* and reverse-*TTCGCCTTTCCCTCACGGTACT* (where Y is C or T and R is A or G). The PCR "master mix" consisted of 1 x PCR buffer, 2.0 mM magnesium chloride, 150 µM of dNTPs, 1.0 µM of each primer and 2 µl of template in a total volume of 50 µl. The PCR conditions consisted of: 95°C for 5 min; [95°C for 15 s/ 55°C for 15 s/ 72°C for 15s] x

25 cycles; [95°C for15 s/ 65°C for 30 s/ 72°C for 15s] X 25 cycles and a final extension at 72°C for 7 min. The PCR products were detected by electrophoresis in 1% agarose gels containing 0.5% ethidium bromide and visualization by UV illumination. A 1 kb DNA (Invitrogen) size marker was included.

4.3 Results

Restriction fragment length polymorphisms (RFLP) generated by Southern hybridization are shown in Figure 4.1. The data in Table 4.1 are a summary of the different groups of fingerprinting profiles generated by the two restriction enzymes. Five isolates of *Map,* FR2616, AA3814, EA4146, EQ2356 and 4200 that originated from 5 geographical regions showed three different fingerprinting patterns using *Bst*EII or *Pvu*II restriction digests. Group 1 of the *Bst*EII digests consisted of EQ2356, FR2616, and AA3814; groups 2 and 3 have only one isolate in each group. They are EA4146 and 4200, respectively. The two reference strains ATCC 43544 and ATCC 19698 also shared the *Bst*EII group 1 pattern. However, the *Pvu*II restriction digest pattern differentiated the reference strain ATCC 43544 into a separate group, designated as Group 4. No changes in the groupings were observed with respect to the other isolates. A recent study by Motiwala *et al.,* (2003) showed a high degree of genetic similarity among bovine *Map* isolates regardless of geographic origin.

A. *Bst*EII restriction endonuclease digest of *Map* DNA

B. *Pvu*II restriction endonuclease digest of *Map* DNA

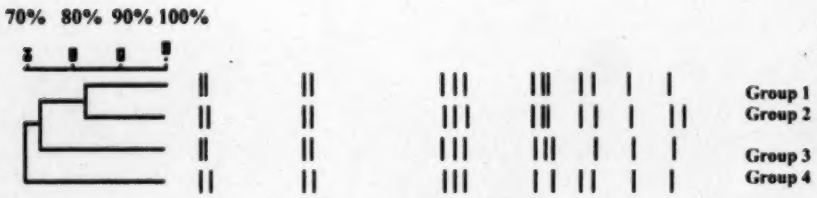

Figure 4.1. RFLP profiles and dendrogram of *Map* strains isolated from different geographical regions. Different *Map* strains, FR2616, AA3814, EA4146, EQ2356, 4200, ATCC 43544 and ATCC 19698 were restricted by *Bst*EII in A and *Pvu*II in B, followed by Southern hybridization with a probe generated by IS*900*.

Table 4.1. Groups of fingerprinting profiles of *Map* strains generated by DNA restricted fragments length polymorphism (RFLP) with two restriction enzymes.

Groups	**Restriction enzymes**	
	***Bst*EII**	***Pvu*II**
1	EQ2356, FR2616, AA3814, ATCC 43544, ATCC 19698	EQ2356, FR2616, AA3814, ATCC 19698
2	EA4146	EA4146
3	4200	4200
4		ATCC 43544

The average yolk volume and concentration of IgY purified using the EggSTRACT® kit based on analysis of 100 eggs was 5.55 ml and 5.75 mg/ml per egg, respectively. Polyacrylamide gel analysis of 25 purified IgY preparations showed two major bands (Figure 4.2). The molecular weight of the major band corresponds to the IgY standard fragment (MW=180 kDa) indicating the success of the extraction; however, there was also a minor contaminating band with a molecular weight of 65 kDa as well as other even more minor bands less than 180 kDa.

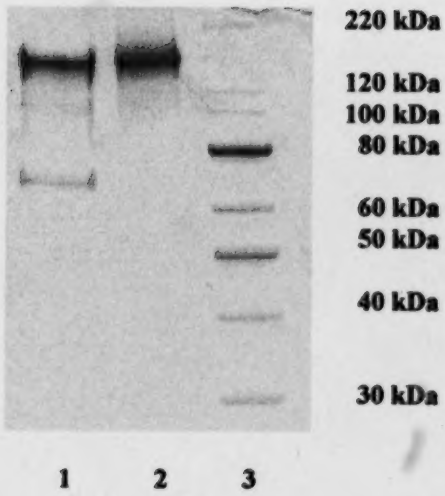

Figure 4.2. PAGE gel analysis of IgY prepared from chicken egg yolk. Lane 1: IgY purified by the EggSTRACT® kit from eggs collected from chicken immunized with heat-inactivated *Map*. The purity of the preparation was compared with a Standard IgY as shown in lane 2. Lane 3: MagicMark™ XP protein standards. The molecular weights of the marker bands are indicated.

There was no growth of heat- or formalin-inactivated *Map* observed on Herrold's egg yolk medium (HEYM) slants after 20 weeks of incubation. OD values at A_{660} of the IgY from the egg yolks of negative control chickens were less than 0.10 in the ELISA test. Samples were classified as positive for *Map* antibodies if the OD in the ELISA test was at least 2 x greater than the OD of the negative control ($\geq$0.20). The titer was defined as the highest dilution of the sample yielding a positive ELISA result. IgY responses of the immunized chickens to formalin- and heat-inactivated *Map* are shown in Figure 4.3. After a booster immunization at week 3 with formalin inactivated antigen, there was an immediate rise in the antibody titer reaching a maximum at weeks 5 and 6. By week 7, the antibody level dropped but administration of a second booster at this time resulted in an immune response that was immediate and reached a higher peak than previously recorded. Once again, the antibody titer slowly decreased over a period of 6 weeks. A similar observation occurred in the antibody response curve when the heat-inactivated antigen was injected into the chickens. Although the OD_{660} readings were consistently higher with the IgY produced from heat-inactivated *Map* than the formalin-inactivated *Map*, no difference was detected in the titers. The titers at 5 to 6 weeks and 8 to 9 weeks were 1/6,400 and 1/12,800, respectively, for both types of immunogens. IgY produced from heat-inactivated *Map* was chosen for the subsequent immunocapture experiments.

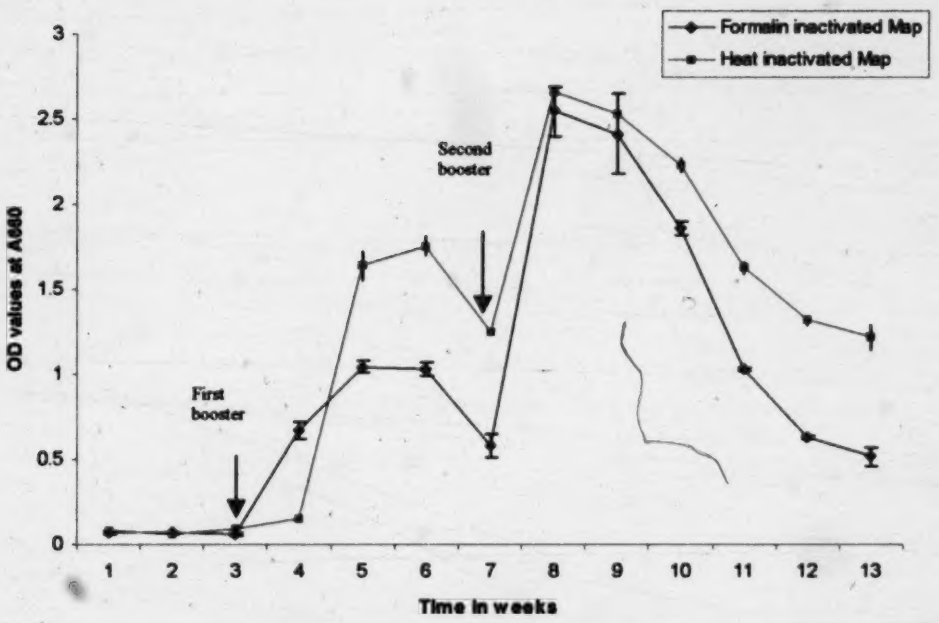

Figure 4.3. IgY response to the immunization of chicken using heat- and formalin-inactivated *Map* over a period of 13 weeks. Vertical bars indicate standard deviation. OD values represent the mean of 5 samples.

Agglutination on the slide was difficult to interpret because the bacterial cells tended to clump before the test was performed. Immunofluorescence was a better assay

to determine the specificity of the IgY. Positive immunofluroescence was observed at 1:50, 1:100, 1:200, 1:500, 1:750 and 1:1000 dilutions with *Map* strains FR2616 and ATCC 19698 but not with *M. avium, M. gordonae, M. intracellulare*, BCG, or *M. tuberculosis* H37Ra. Initial immunofluorescent studies showed cross-reactivity of the IgY with the *M. avium* strain at the two low dilutions (1:50, 1:100) but no fluorescence was observed with the same dilutions for the other *Mycobacterium* species. After the pre-adsorption with *M. avium* cells, none of the dilutions of IgY used for the immunofluorescent assay cross reacted with *M avium* cells but there was a slight decrease in fluorescence with *Map* when incubated with 1:1,000 dilution of IgY.

M. tuberculosis, *M. intracellulare*, BCG, *M. gordonae*, *Map* and *M avium* were included in the capture study. Only *Map* was captured by the IgY as shown in Figure 4.4A using primers directed to the 23S rRNA and further confirmed by PCR using primers J5A and J5B directed to the insertion element IS*900* (Fig 4.4B). DNA was successfully extracted from all of the noncaptured *Mycobacterium* species as shown by the PCR results using the 23S rRNA primers (Figure 4.4C). In addition, six strains of *Map* that were not included in the immunization of the chickens (ATCC 19698, 11520-5, 12258, 11992, ER2945Y162, ATCC 43544) and the four strains used to inject the chickens (FR2616, AA3814, EA4146 and EQ2356) were all positive by PCR using primers J5A and J5B following the IgY immunocapture assay (Figure 4.5). Although strain 4200 that belonged to RFLP Group 3 was not included in Figure 4.5, it too was captured.

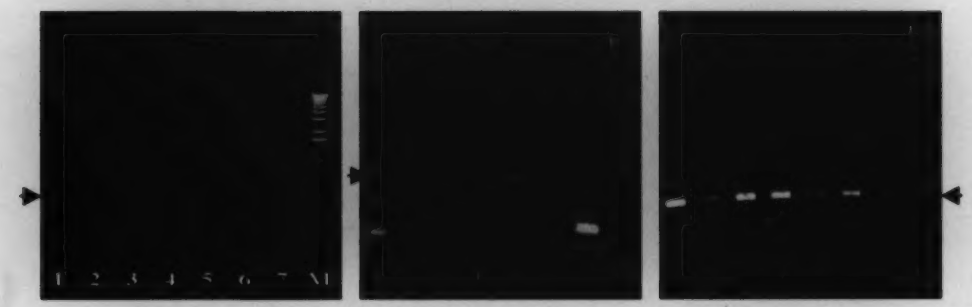

Figure 4.4. PCR results with immunocapture using IgY isolated from egg-yolk previously immunized with heat-inactivated *Map*. Lane 1: *M. tuberculosis* H37Ra; Lane 2: *M. intracellulare;* Lane 3: BCG; Lane 4: *M. gordonae*; Lane 5: *Map,* Lane 6: *M. avium;* Lane 7: negative control. M: 1 kb marker. The bacterial cells were subjected to immunocapture in A and B. PCR primers were directed to the 23S rRNA sequence in A (amplicon size: 495 bp) and IS*900* in B (amplicon size: 298 bp). Cells were not captured in C and the primers for the PCR assay were from the 23S rRNA region (amplicon size: 495 bp). The arrow indicates the location of the amplicon (▶).

Figure 4.5. Different strains of *Map* were captured by IgY and PCR was performed using primers J5A and J5B. The size of the amplicon is 298bp. Lanes 1 to 11: *Map* strains AA3814, ATCC 19698, FR2616, 11520-5, 12258, 11992, ER2945Y162, EA4146, ATCC 43544, EQ2356, negative control, respectively. M:1 kb molecular size marker.

4.4 Discussion

In Chapter 3, it was shown that screening for *Map* in cattle feces using PCR encountered major problems because of inhibition and that a 1:100 dilution of the extracted DNA was required. As a result, samples of feces with low bacterial loads could produce false negative results. To increase the sensitivity of PCR by eliminating the need for diluting the template DNA, production and characterization of IgY for an immunocapture assay was investigated. Applications using IgY for detecting pathogens such as *Salmonella* (Opitz *et al.*, 1993; Yokoyama *et al.*, 1998a, b; Yoshiko *et al.*, 1996), *E. coli* (Ikemori *et al.*, 1992; Imberechts *et al.*, 1997; O'Farrelly *et al.*, 1992; Sunwoo *et al.*, 2002; Yokoyama *et al.*, 1992, Wiedemann *et al.*, 1991), *Streptococcus* (Chang, 1999; Hamada *et al.*, 1991; Hatta *et al.*, 1997), *Staphylococcus* (Yoshiko *et al.*, 1996), rotavirus (Kuroki *et al.*, 1993, 1997) and coronavirus (Ikemori *et al.*, 1997) have been published. In this investigation, *Map* induced an effective immune response in chickens. A "pool" consisting of four strains of *Map* from different geographic regions of the province of Alberta was used to immunize the chickens. Because *Map* is a potential pathogen for humans and chickens, inactivation of the bacteria was necessary. Inactivation by heat or by 10% formalin had no effect on the ability of the purified IgY to recognize live *Map* cells as illustrated by the immunofluorescence assay and immunocapture PCR. Furthermore, the ELISA titers were identical when both types of heat and formalin inactivated antigens were used for immunization.

The immune response detected by OD values at A_{660} to cells that were inactivated by heat was higher than to those inactivated by formalin. The bacterial

suspensions for immunization was adjusted to a MacFarland #1 standard and this crude measure of cell numbers could explain the slight differences in immune response between the two methods of inactivation used. The secondary response, after booster injection, was much higher than the primary response independent of the type of antigen used for immunization. The IgY was specific and there was no cross-reactivity observed with other closely related *Mycobacterium* species except *M. avium*. Adsorption of the IgY by *M. avium* cells, eliminated the non-specific binding without affecting the binding of the IgY to the 10 different *Map* strains tested, independent of whether they had been included as immunogen for the immunization program. However, a slight decrease in binding efficiency of the antibody to *Map* was observed after pre-adsorption as indicated by the result of the fluorescent assay using 1:1000 dilution of the antibody. This can be explained by loss of IgY bounded to *M. avium* during the pre-adsorption step. The IgY preparation using the EggSTRACT®kit can yield high quality and quantity antibody. A contaminating band, with a molecular weight at 65 kDa and few minor bands were detected by PAGE but it did not have an effect on the binding of IgY to *Map*.

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5. Development of an immunocapture PCR assay for the diagnosis of Johne's disease in cattle

5.1 Introduction

Nucleic acid amplification has been widely used as a tool in diagnostic laboratories to detect a variety of pathogens. Several PCR assays have been developed (Collins *et al.*, 1993 a, b; Dell'Isola *et al.*, 1994; Secott *et al.*, 1999; van der Giessen *et al.*, 1992; Vary *et al.*, 1990) for the detection of *M. avium* subsp *paratuberculosis* (*Map*). However, the gold standard for the diagnosis for Johne's disease is still based on culture of bovine stool samples and the results may take 8 to 20 weeks before they are available. The lag time in determination of the herd's status to Johne's disease can result in further spreading of the infection to other susceptible animals on the farm. However, by using PCR as a detection method of *Map* on bovine feces or milk, the result can easily be obtained within 24 h. Unfortunately, inhibitory factors such as blood, bile, complex carbohydrates, heavy metals that are present in fecal specimens (Al-Soud and Radstrom, 2000; Holland *et al.*, 2000; Krause *et al.*, 2001) or the whey fraction of milk which is high in calcium ions (Grant *et al.*, 2000), may co-extract with the target DNA and interfere with the activity of the polymerase enzyme used in the PCR assay. In order to circumvent this problem, immunoseparation (IMS) technique has been used to capture the organism of interest and through serial washings remove the inhibitors before the DNA extraction step.

IMS utilizes small super-paramagnetic particles or beads coated with antibodies against the surface antigens of eukaryotic or prokaryotic cells. The super-paramagnetic particles are attracted to each other in a magnetic field but they are nonmagnetic as soon as the magnetic field is removed. The magnetic spheres most commonly used are made by mixing Fe^{2+} with monosized porous polymer beads. A new polymer surface layer will then close the pores and keep the iron inside the particles (Ugelstad *et al.*, 1992). An inert surface that does not bind to biological components other than the specific target is most desirable and chemical composition of the surface of the particle is one of the important factors determining the success for IMS technique.

In clinical diagnostic microbiology, IMS has been used as a primary tool to capture the pathogens from specimens such as feces, food, blood followed by further identification by microscopy (Lund *et al.*, 1988), culture (Christensen *et al.*, 1992; Fratamico *et al.*, 1992; Johne *et al.*, 1989, Kapperud *et al.*, 1993; Morgan *et al.*, 1991; Skjerve *et al.*, 1990; Vermunt *et al.*, 1992), hybridization (Lund *et al.*, 1991; Olsvik *et al.*, 1991), PCR (Mason *et al.*, 2001) and sequencing (Hultman *et al.*, 1989; Lewis *et al.*, 1992; Olsvik *et al.*, 1993; Wahlberg *et al.*, 1990a, b).

IMS using rabbit polyclonal antibody was developed to detect *Map* in milk (Djonne *et al.*, 2003a, b; Grant *et al.*, 1998, 2000, 2002) or fecal samples (Mason *et al.*, 2001). By using this technique, low numbers of bacteria present in the sample are concentrated and it also allows the removal of coextracted inhibitory factors through successive washing of the magnetic beads. In Chapter 4, a "pool" of four *Map* strains was injected into the breast muscle of Single Comb White Leghorn chickens and an

immune response was elicited with an antibody titer of 1/12800. The objective of this study is to develop a capture assay using immunoseparation technique for detecting *Map* in bovine feces.

5.2 Materials and methods

5.2.1 *Preparation of IgY for coating of magnetic beads.*

Purified IgY from chicken immunized with heat-inactivated *Map* with ELISA titer of greater than 1/5000 was pooled and used in the following experiments. The ratio of rabbit anti-chicken IgG Maga™ beads to IgY used in the assays were 1 mg : 1500 ng and 1 mg : 6000ng and the reaction between the two components was done at room temperature for 30 min as recommended by the manufacturer (Cortex BioChem, San Leandro, CA). The preparations were designated as Beads A and Beads B preparations, respectively. In addition, an aliquot of purified IgY was coupled to Magnetizable cellulose/iron oxide particles prepared by Cortex BioChem at a concentration of 30 mg/ml (w/v) and was designated MagaCell™-IgY.

5.2.2 *Determination of sensitivity of the PCR assay without immunocapture*

Three field and two reference strains of *Map* (11992, EA4146, 2945, ATCC 12258 and ATCC 19698) were standardized at a wavelength of 550 nm to give an OD corresponding to approximately 2 X 10^7 cells/ml. Dilutions of this standardized cell suspension were made from 10^{-3} to 10^{-7} in 12 mM Tris-HCl, pH 7.4 and 200 µl of each

dilution was extracted with the MagaZorb® DNA isolation kit (Cortex BioChem). The DNA was eluted from the beads in 200 µl of 12 mM Tris-HCl buffer and 10 µl was used as template for PCR.

5.2.3 *Determination of the sensitivity of the PCR assay with SDS treatment followed by immunocapture with different types of beads*

To determine the optimal conditions for the immunocapture assay, the reference strain *Map* ATCC 19698 and *Map* field strain EA4146 were selected for use in the study. Strain EA4146 was in the *Map* pool for the immunization of the chickens; but strain ATCC 19698 was not included in the pool. Restriction Fragment Length Polymorphism (RFLP) analysis (Chapter 4) of these strains showed differences in their fingerprinting profiles and allows the opportunity to compare binding affinity of two different strains of *Map*. A 1 ml volume containing approximately 2 x 10^7 cells was suspended in 0.6% SDS and put on a shaker (Eberback model 6000, Ann Arbor, Michigan) at low speed setting for 30 min. The cells were pelleted by centrifugation at 13,000 x *g* and washed with 1 ml of 12 mM Tris-HCl, pH 7.4. Following the wash, the cells were resuspended in 1 ml of the same buffer. A 200 µl sample was centrifuged at 13,000 x *g* for 3 min and the cell pellet was resuspended in 990 µl of phosphate buffered saline (PBS) at pH 7.4. A 10 µl volume of Beads A or Beads B was added and the tubes were held at 37°C for 1.5 h. After this step, both types of beads were washed five times with 12 mM Tris-HCl and resuspended in 0.1% SDS at 23°C for 15 min to release the bacteria from the beads. The reaction tubes were placed in the MPC®-S unit (DyNal

Biotech LLC) for 3 min and DNA was extracted from the supernatant using the MagaZorb® (Cortex BioChem) DNA isolation kit. DNA was then diluted from 10^{-1} to 10^{-5} and 10 µl was used as template in a PCR assay volume of 50 µl. For the control, the same concentrations of cells were treated with 12 mM Tris-HCl, followed by the capture assay. For the non-capture assay, DNA was also extracted from the SDS-treated and non-treated cells, diluted and used as template for PCR.

5.2.4 *Determination of volume of IgY beads, incubation time and temperature requirement for the immunocapture assay*

Beads A suspension in the amount of 10, 20, 30 or 40 µl was added to a 1.5 ml micro centrifuge tube containing 2 x 10^4 *Map* cells (ATCC 19698 or EA4146) in a total volume of 1 ml PBS. The mixture was held at 37°C for 1.5 h followed by the washing and extraction steps as described above. The DNA was diluted 1:10 and 1:100 and 10 µl from each dilution as well as the undiluted DNA were used as template for PCR.

To determine the incubation time and temperature required for the immunocapture assay, the same number of cells of these strains was reacted with the optimized amount of Beads A and the mixture was held at 37°C and 23°C for 15, 30, and 60 min using a MyLab ™ Rotamix SLRM1 (Seoulin, MJSBiolynx Inc, Brockville ON).

The purified IgY was sent to Cortex BioChem to be coupled onto magnetizable cellulose/iron oxide particles, referred to as MagaCell™-IgY in this study. Similar optimization was carried out as described above for Beads A and B. To test for specificity of MagaCell ™-IgY beads, *Map* (ATCC 19698), *M. avium, M. gordonae, M.*

intracellulare, BCG and *M. tuberculosis* H37Ra cells were added to the beads and held at 37°C for 1 h with rotation. After 5 washes with 1 ml of PBS, the beads were placed on a microscope slide and air dried inside a biosafety cabinet. The slide was flooded with 5% phenol in 70% ethanol, fixed for 30 min and then stained with Ziehl-Nielsen stain. The slide was examined under a light microscope.

5.2.5 *Optimized immunocapture assay on fecal samples spiked with MAP*

Bovine feces, previously confirmed to be negative for *Map* by culture and stored at –80°C were provided by Agri-Food Laboratories Branch, Alberta Agriculture, Food and Rural Development, were used for the spiking experiments. The strains used were *Map* field strains 11992, EA4146 and 2945 and reference strains ATCC 12258 and ATCC 19698. One gram of frozen feces was thawed overnight at 4°C and mixed with 1 ml of a *Map* strain at a concentration of 10^3, 10^4 or 10^5 cells. An unspiked fecal sample was included as a negative control. Each sample was added to a conical centrifuge tube containing 24 ml of 0.6% (w/v) SDS in water. The suspension was mixed by vortex for 1 min and was placed on a horizontal shaker (Eberback model 6000, Ann Arbor, Michigan) for 30 min set on low speed. After 30 min of standing at 23°C, to allow the particulate matter to settle, 20 ml of the supernatant was removed and centrifuged at 2380 x *g* for 30 min. The supernatant was discarded and 24 ml of sterile, purified water was added to the pellet, mixed by vortex for 1 min and centrifuged 2380 x *g*. The supernatant was discarded and washing was repeated. After centrifugation, the pellet was resuspended in 1 ml of PBS, pH 7.4. These spiked samples and an unspiked

negative control were captured by Beads A and MagaCell™-IgY for comparison. DNA was extracted using the MagaZorb® DNA kit (Cortex BioChem) and 10 µl was used as template for PCR. A summary of the optimization for both cells and bovine feces spiked with cells is shown in Figure 5.1.

5.2.6 *PCR and detection of amplicons*

PCR was performed using primers J5A and J5B (Hermon–Taylor *et al.*, 2000) with conditions described in section 3.2.5. The amplicons were detected by electrophoresis in 1% agarose gels containing 0.5% ethidium bromide and visualized by UV illumination. A 1 kb DNA size marker was included in each gel (Invitrogen Canada Inc.).

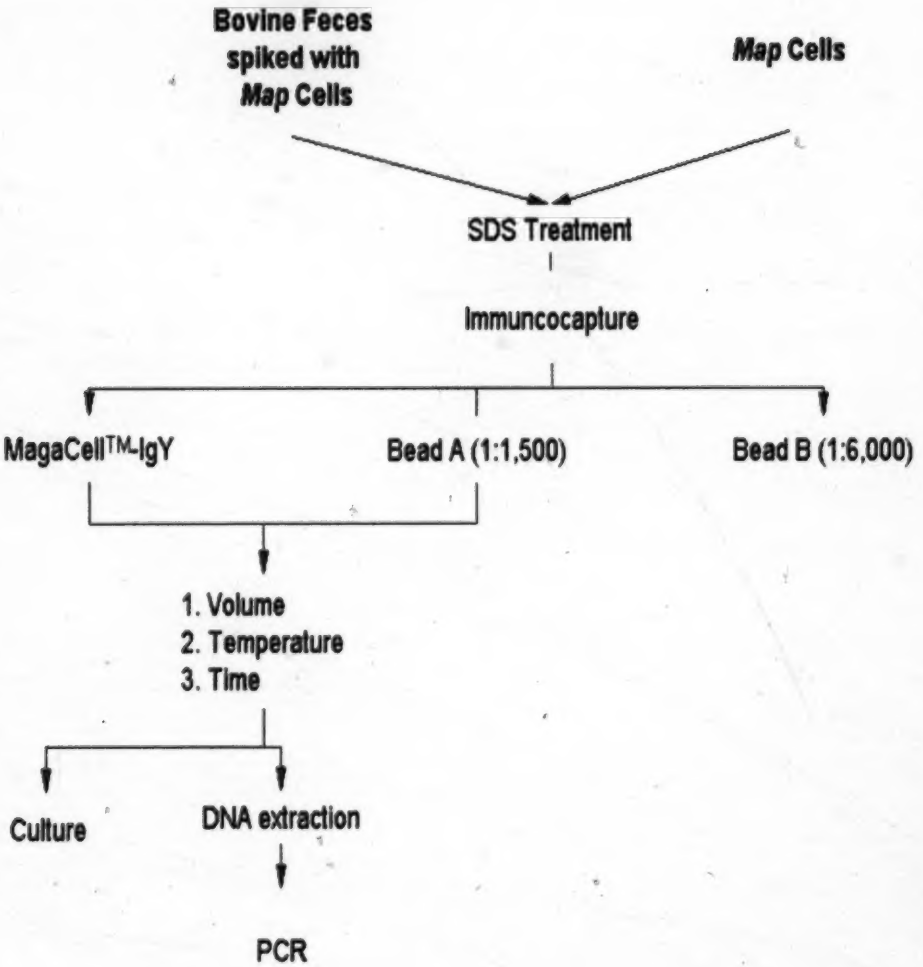

Figure 5.1. Flowchart showing the optimization for the immunocapture assay

5.3 Results

The titration of three field and two reference strains of *Map* (11992, EA4146, 2945, ATCC 12258 and ATCC 19698) gave similar endpoint results and data for two strains (*Map* 11992 and ATCC 19698) are shown in (Figure 5.2 A and B). The PCR sensitivity was detected at the cell dilution of 10^{-6}, which is equivalent to a cell count of approximately 1 cell/PCR assay with 10 µl template DNA.

Ten microliters of Beads A or B were used in the initial experiment to capture 2 x 10^6 *Map* cells for both strains EA4146 and ATCC 19698. Comparison between the two preparations of capture beads showed that Beads A (1 mg : 1500 ng) was better than the Beads B (1 mg : 6000 ng) with 0.6% SDS treatment as shown by the data in Figure 5.2, lanes 1 to 3 and 7 to 9. In the absence of SDS, however, the endpoint is the same regardless of the type of immunobeads used. Results from PCR titration with diluted DNA (10^{-3} to 10^{-5}) extracted from cells without the immunocapture in the presence of SDS (Figure 5.3, lanes 13 to 15) or absence of SDS (Figure 5.3, lanes 16 to 18) were identical. The same results were obtained with *Map* strain EA4146.

The PCR endpoint titrations with extracted DNA at undiluted, 1:10 and 1:100 dilutions from bacteria captured with different amounts of Beads A are illustrated in Figure 5.4. The intensity of the PCR band increases as the volume of Beads A used for immunocapture increases from 10 µl to 30 µl (Figure 5.4, lanes 1 to 9). No difference in the intensity of the PCR band was observed when 30 µl or 40 µl of Beads A were used for the capture (Figure 5.4, lanes 7 to 12). However, when the same experiment was run with 2 x 10^3 cells/g of bovine feces, inconsistency was observed in the endpoint PCR

titrations. Increasing the cell concentration to 2 x 10^4 /g in the spiking experiment with the addition of 40 µl of Beads A was required to obtain a positive PCR result (Figure 5.5 lane 10). Adding less than 40 µl of Beads A for immunocapture failed to give a positive PCR result. Correspondingly, PCR results of the spiked fecal samples without immunocapture revealed that a 1:100 dilution of the extracted DNA was required for amplification to occur (Figure 5.5, lanes 13 and 14). Although extensive washing was performed after the capture, some unknown inhibitors were co-extracted with the target and thus resulted in inhibiting the amplification of the internal control as shown in lanes 1, 4, 7 and 10 of Figure 5.5.

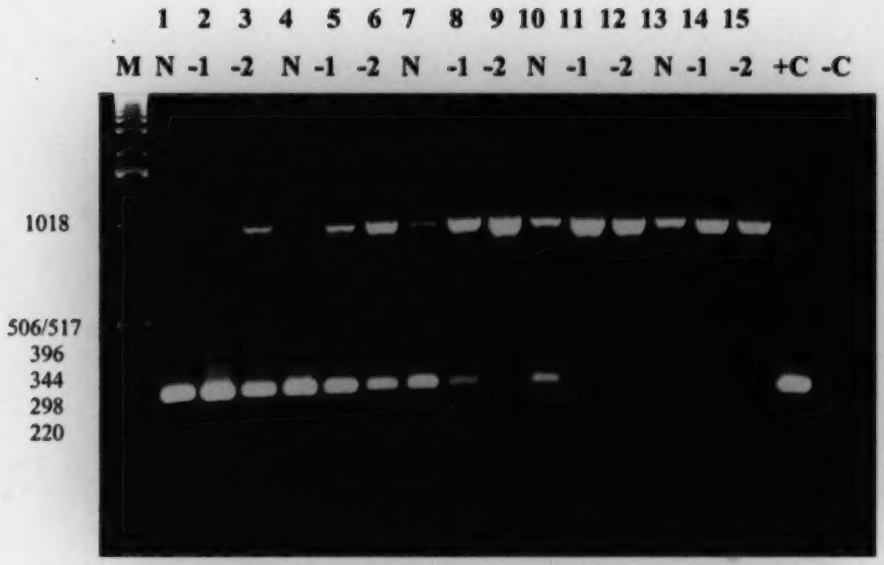

Figure 5.2A. PCR endpoint titration of *Map* reference strain ATCC 19698. The volume of target DNA used for PCR assays was 10 µl. Lanes 1 to 3: DNA extracted from 4,000 cells run undiluted (N), 1:10 dilution (-1) and 1:100 dilution (-2). Lanes 4 to 6: DNA extracted from 400 cells run N, -1 and –2. Lanes 7 to 9: DNA extracted from 40 cells run N, -1 and –2. Lanes 10 to 12: DNA extracted from 4 cells run N, -1 and –2. Lanes 13 to 15: DNA extracted from 1 or less than 1 cell run N, -1 and –2. Lane +C is internal control and positive control DNA (ATCC 19698). Lane -C is negative water control. Lane M: 1 kb molecular weight marker.

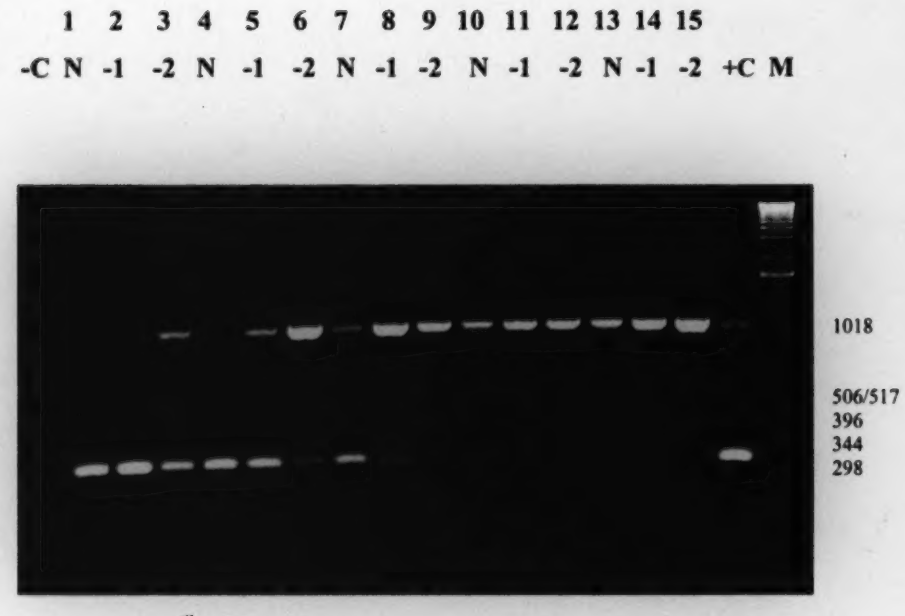

Figure 5.2B. PCR endpoint titration of *Map* strain 11992. The volume of target DNA used for PCR assays was 10 µl. Lanes 1 to 3: DNA extracted from 4,000 cells run undiluted (N), 1:10 dilution (-1) and 1:100 dilution (-2). Lanes 4 to 6: DNA extracted from 400 cells run N, -1 and –2. Lanes 7 to 9: DNA extracted from 40 cells run N, -1 and –2. Lanes 10 to 12: DNA extracted from approximately 4 cells run N, -1 and –2. Lanes 13 to 15: DNA extracted from 1 or less than 1 cell run N, -1 and –2. Lane +C is internal control and positive control DNA (ATCC 19698). Lane -C is negative water control. Lane M: 1 kb molecular weight marker.

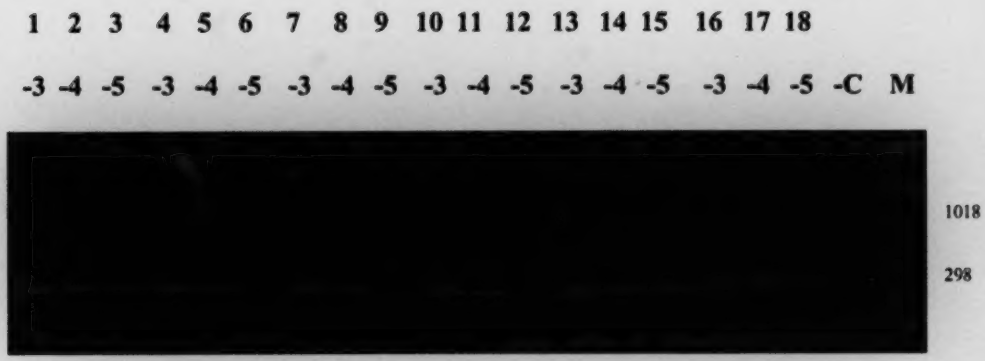

Figure 5.3. Comparison of immunocapture with Beads A and Beads B in the presence and absence of 0.6% SDS. Dilutions of the extracted DNA (ATCC 19698) from 10^{-3} to 10^{-5} are indicated as -3, -4 and -5. Lanes 1-3: Beads A with SDS treatment. Lanes 4 to 6 Beads A without SDS treatment. Lanes 7 to 9: Beads B with SDS treatment. Lanes 10 to 12: Beads B without SDS treatment. Lanes 13 to 15: no immunocapture with SDS treatment. Lanes 16 to 18: no immunocapture without SDS treatment. Lane -C is the negative water control and lane M is the 1kb molecular weight marker.

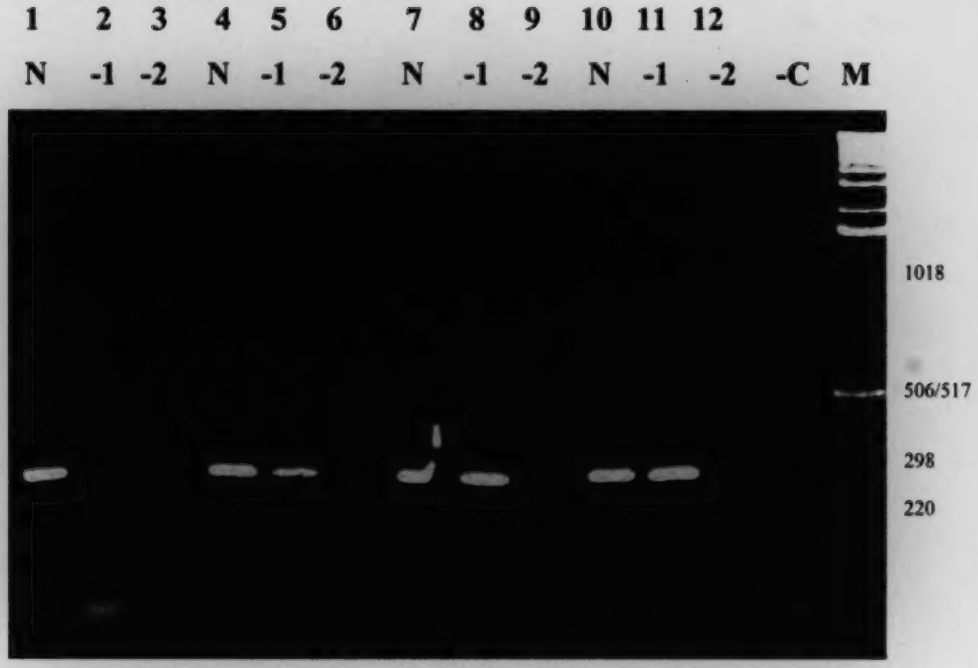

Figure 5.4. Determination of Beads A required for immunocapture using *Map* strain ATCC 19698. DNA was run undiluted (N), 1:10 dilution (-1) and 1:100 dilution (-2) for each Beads A volume used. Lanes 1 to 3: 10 µl Beads A. Lanes 4 to 6: 20 µl Beads A. Lanes 7 to 9: 30 µl Beads A. Lanes 10 to 12: 40 µl Beads A. Lane -C negative water control. Lane M: 1 kb molecular weight marker.

Figure 5.5. Determination of Beads A required for immunocapture using *Map* strain ATCC 19698 (2 x 10^4 cells) spiked with 1g of bovine feces. DNA was run undiluted (N), 1:10 dilution (-1) and 1:100 dilution (-2) for each Beads A volume used. Lanes 1 to 3: 10 µl Beads A. Lanes 4 to 6: 20 µl Beads A. Lanes 7 to 9: 30 µl Beads A. Lanes 10 to 12: 40 µl Beads A. Lanes 13 to 14: no immunocapture. Lane +IC: internal control DNA. Lane +C Positive control DNA (ATCC 19698). Lane -C negative water control. Lane M: 1 kb molecular weight marker.

The PCR titration results showed that there was no difference in the capture efficiency when it is done at 23°C or 37°C for 15, 30 or 60 min using 2 x 10^4 *Map* cells/ml (ATCC 19698 or EA4146) and 40 µl Beads A in the reaction assay. The data with 23°C incubation at three different holding times is illustrated in Figure 5.6. Identical parameters for incubation temperature and time with the same amount of Beads A tested on both strains of *Map* cells gave identical endpoint PCR result. In Figure 5.7, the data showed that 40 µl Beads A at 23°C for 15 min was sufficient to capture *Map* strain ATCC 19698 (2 x 10^4 cells) in the presence of bovine feces.

The third type of beads included in this study was MagaCell ™-IgY, the direct coupling of IgY to the magnetizable cellulose/iron oxide particles customized by Cortex BioChem. The amount of IgY bound to the beads was approximately 18 mg/gram of particles and the concentration was at 3 x 10^8 particles/ml. The specificity of MagaCell™-IgY beads tested against *Map* reference strain ATCC 19698, *M. avium, M. gordonae, M. intracellulare*, BCG, *M. tuberculosis* H37Ra in an immunocapture assay showed that only the *Map* reference strain ATCC 19698 was bound to MagaCell™-IgY beads (Figure 5.8) as shown by Ziehl-Nielsen staining.

Because the immunocapture assay was designed for use with bovine feces, the optimization of MagaCell™-IgY was based on the previous results from Beads A preparation with feces spiked with *Map* that had been pretreated with 0.6% SDS. The volumes of MagaCell™-IgY used for the optimization study were 5, 10, 15 and 20 µl with concentrations of IgY ranging from 2.7 µg to 10.8 µg per immunocapture assay. The data in Figure 5.9 illustrated that there was no difference in the PCR detection level

with the various amounts of IgY after immunocapture assay at 23°C for 15 min. Although a faint band was observed in lane 6 at 10^{-2} dilution of DNA captured by 10 µl of MagaCell™-IgY, it was most likely due to sampling error and was not seen when repeated.

The efficiency of Beads A and MagaCell™-IgY capture assays was also compared using identical conditions for time and temperature with bovine feces spiked with *Map* strain ATCC 19698 at concentrations of 10^3, 10^4 and 10^5 cells/g. In the reaction using 10 µl of MagaCell™-IgY for the immunocapture, the detection level was the same (Figure 5.10) compared with 40 µl of IgY-Beads A (Figure 5.7) for the capture. However, no inhibition was observed with immunocapture assay using MagaCell™-IgY. Identical results were obtained with *Map* field strains 11992, EA4146, 2945 and reference strain ATCC 12258.

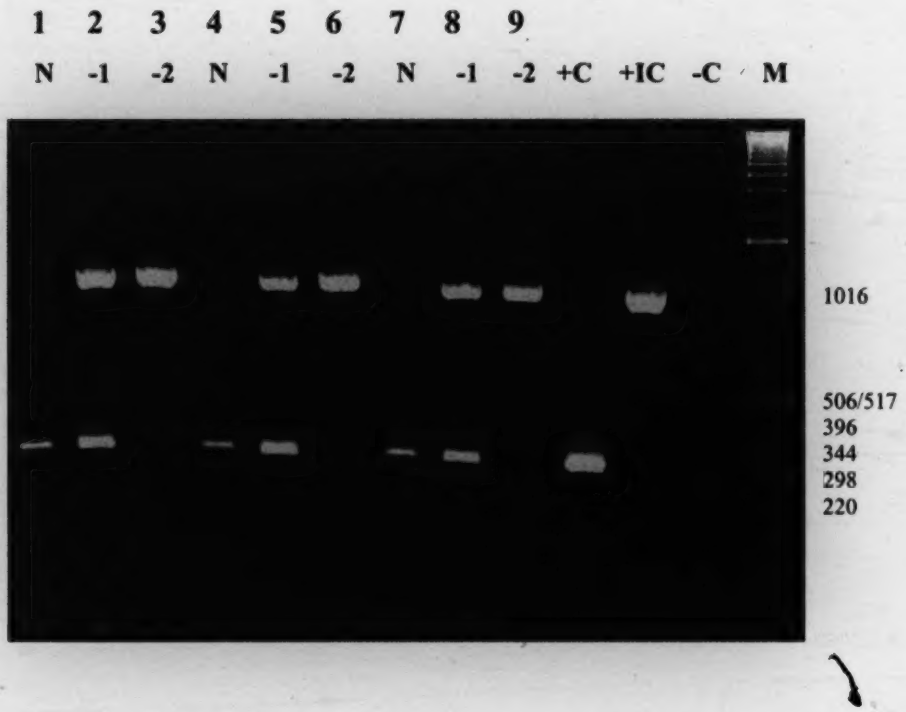

Figure 5.6. Determination of incubation time for immunocapture using Beads A with *Map* strain ATCC 19698 at 23°C. DNA was run undiluted (N), 1:10 dilution (-1) and 1:100 dilution (-2) with 40 µl of Beads A for the immunocapture. Lanes 1 to 3: 15 min. Lanes 4 to 6: 30 min. Lanes 7 to 9: 60 min. Lane +IC: internal control DNA. Lane +C Positive control DNA (ATCC 19698). Lane -C: negative water control. Lane M: 1 kb molecular weight marker.

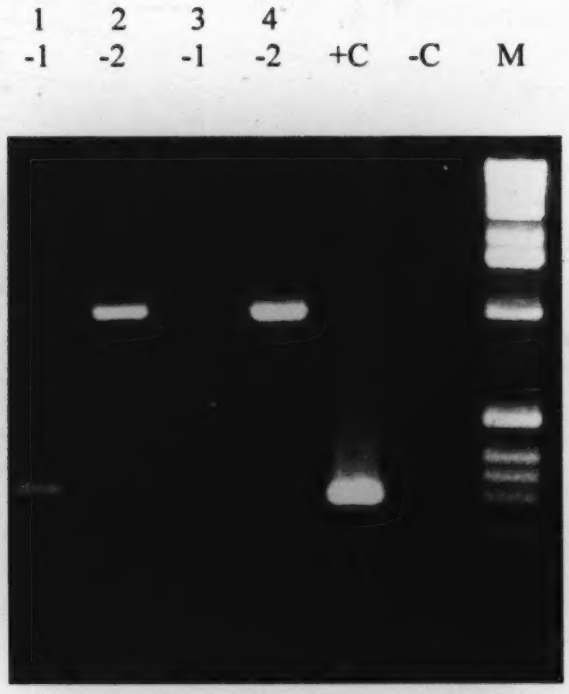

Figure 5.7. PCR result using DNA extracted from bovine feces spiked with *Map* strain ATCC 19698 after captured by 40 µl Beads A at 23°C for 15 min. DNA was run 1:10 dilution (-1) and 1:100 dilution (-2). Lanes 1 and 2: with immunocapture. Lanes 3 to 4: negative control bovine feces. Lane +C: Positive control DNA (ATCC 19698). Lane -C: negative water control. Lane M: 1 kb molecular weight marker.

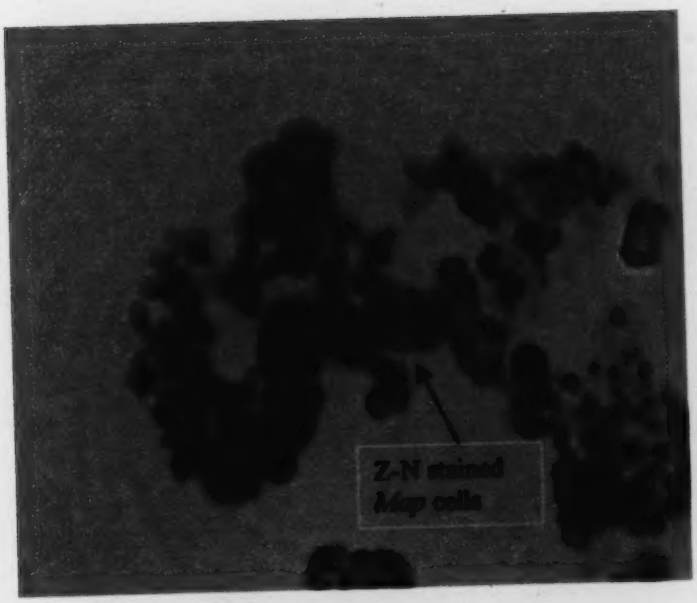

Figure 5.8. Ziehl-Nielsen (Z-N) staining of immunocaptured *Map* ATCC 19698 cells by MagaCell ™-IgY (x 500)

Figure 5.9. Determination of MagaCell ™-IgY volume required for immunocapture using 10^4 cells/g of *Map* strain ATCC 19698 spiked in bovine feces at 23°C for 15 min. DNA was run undiluted (N), 1:10 dilution (-1) and 1:100 dilution (-2) for each MagaCell ™-IgY volume used for capture. Lanes 1 to 3: 5 µl MagaCell ™-IgY. Lanes 4 to 6: 10 µl MagaCell™-IgY. Lanes 7 to 9: 15 µl MagaCell ™-IgY. Lanes 10 to 12: 20 µl MagaCell™-IgY. Lane +C: Positive control DNA (ATCC 19698). Lane -C: negative water control. Lane M: 1 kb molecular weight marker.

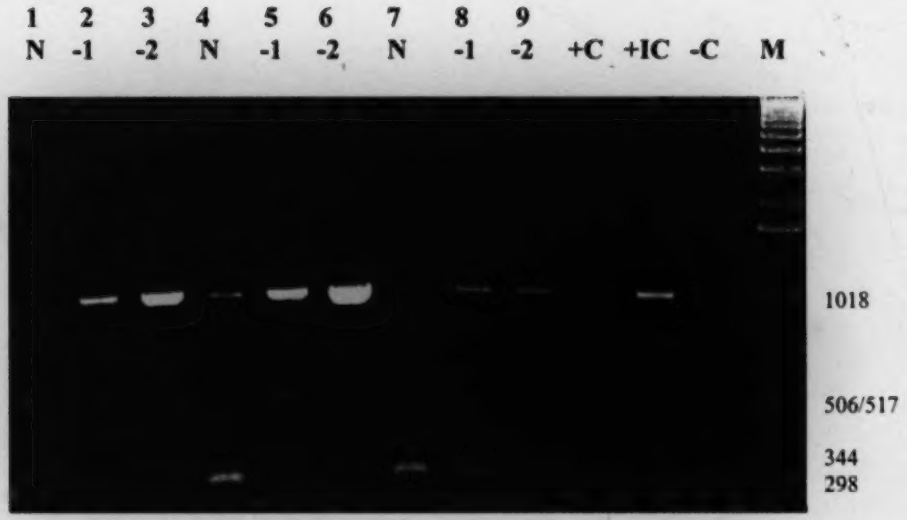

Figure 5.10. Bovine feces spiked with *Map* ATCC 19698 at 2 x 10^3 cells/g (lanes 1 to 3), 2 x 10^4 cells/g (lanes 3 to 6) or 2 x 10^5 cells/g (lanes 7 to 9) were captured by 10 µl of MagaCell™-IgY beads. PCR endpoint titration was performed with extracted undiluted and tenfold dilutions of DNA from 10^{-1} to 10^{-2} indicated as N, -1 and –2. Lane +C: Positive control DNA (ATCC 19698). Lane +IC: positive internal control. Lane -C: negative water control. Lane M: 1 kb molecular weight marker.

5.4 Discussion

Different studies have been published on the utilization of IgG purified from rabbits to be used for immunocapture assays for *Map* (Djonne *et al.*, 2003a, b; Grant *et al.*, 2000; Mason *et al.*, 2001) but this is the first study to show the utility of IgY prepared from chicken eggs as the antibody for IMS (immunoseparation) for capturing *Map*. The detection of pathogens by IMS has been used in conjunction with culture enrichment to increase the cell numbers prior to immunocapture (Ellingson *et al.*, 2004; Holland *et al.*, 2000). However, due to the slow growing nature of *Map*, this approach was not included in our study. The sensitivity of the amplification assay with *Map* cells using PCR with IS*900* primers is at the level of 1 cell per assay compared with IMS-PCR, which is at approximately 10 cells, and 100 cells in the presence of bovine feces. In a study by Mason *et al.* (2001), it was reported that there was a decrease in sensitivity with IMS-PCR of *Map* in the presence of sheep feces compared with no IMS-PCR with the same cell suspension spiked with bovine feces. This increase in sensitivity most likely is due to the presence of free DNA from degraded *Map* cells compared with the IMS technique. Only the intact organisms would bind to the specific antibody coated on the magnetic beads.

Bovine feces are a very heterogeneous and complex and decrease in the sensitivity in IMS-PCR with this type of suspension may be due to several reasons. The presence of non-specific material can interfere with the binding site of *Map* to the antibody. It was substantiated by our data that a higher volume of IgY beads was required to capture the same number of *Map* cells in the presence of bovine feces. The

immunocapture beads may also be trapped in between the bovine feces and with serial washes of the beads during the IMS procedure, the beads could be lost in the process and thus losing the bacteria. Furthermore, our data is also in aggreement with the findings of Mason *et al.* (2001) that the sensitivity of IMS-PCR was at 10^4 viable *Map* cells/g of spiked feces using PCR primers directed to IS*900* sequence.

Bead coating methods were also evaluated in this investigation. Our data showed that direct coating of IgY (MagaCell ™-IgY beads) onto the beads did not show increase in the capability of immunocapture as demonstrated by PCR endpoint titration when compared with the indirect coating of the beads (Beads A) via an antibody linker. A similar study was undertaken by Mason *et al.* (2001) in which antibody to *Map* was directly coated onto the immunomagnetic beads using tosyl chemistry or indirectly via an antibody linker. Their results also showed no difference in the capability of capture with the two types of beads. Our direct coating of antibody used cyanogen bromide activation on the beads and we observed no inhibition in any of the PCR assays compared with the indirect coating of antibody beads by antibody linker for capturing *Map* in the presence of bovine feces. This was not discussed in the investigation by Mason and his collaborators.

A 0.6% SDS treatment was examined in our study and it seemed to improve the immunocapture assay for *Map* cells. This detergent may dislodge the aggregation of cells from the fecal material and facilitate the binding of the bacteria to the beads. Due to the aggregated nature of the *Map* cells and the fact that they are intracellular, this additional step may help to release them from within eukaryotic cells and separate the

bacteria into individual cells, thus increasing the number accessible to the beads and facilitating their binding to the immobilized IgY molecules. The binding capacity was further improved with direct attachment of IgY to the magnetic beads. Other detergents such as Tween have been used (Mason *et al.*, 2001) and most likely serve the same purpose.

The IgY used in this study is purified polyclonal antibodies raised against a pool of 4 different heat inactivated *Map* strains to coat the immunocapture beads. Polyclonal antibodies are not as specific as monoclonal antibodies because they are directed to a number of surface antigens of the bacteria rather than a single surface antigen. This can allow the antibody to recognize other *Map* strains that were not included in the original pool for the immunization. However, cross-reactivity with *M. avium* was observed (Chui *et al.*, 2004) and was eliminated after adsorption with *M. avium* cells. Grant *et al.* (1998) also recommended a higher dilution of the IgG to use for the coating of the beads to circumvent the problem of cross-reactivity with other mycobacterium strains.

IMS is a simple technique but quite labor intensive especially when a large number of specimens are to be processed if no automation is available for the washing steps. The beads may be lost during the sequential washing steps when the supernatant is being aspirated. Another potential drawback is that non-specific material can bind to the beads and also be attracted to the magnet so that it is difficult to wash the beads to remove all of the non-specific material. We did not observe any sliding of the beads with the aspiration of the supernatant as observed by Grant *et al.* (1998). The washing

steps were time-consuming, labor-intensive and required special attention and care to avoid aspirating the beads along with the supernatant.

This chapter demonstrated the use of capture assay by IMS for detecting *Map* in bovine feces. The IgY purified from chicken eggs was used to coat the beads directly by cyanogen bromide inactivation or indirectly by an antibody linker. Using IMS coupled with conventional PCR using gel-based detection, the turn around time for reporting of 10 samples is less than 8 hours. This is much shorter than conventional culture method that requires 8 to 20 weeks before results are available. The detection level with bovine feces spiked with cells was at 100 cells per PCR assay after captured with MagaCell ™-IgY beads. The inhibitory problem for PCR was overcome as illustrated in this chapter but future work is required to improve the sensitivity of the assay before implementation as a routine diagnostic tool for the detection of *Map* in the diseased animals.

5.5 References

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6. Comparison of real-time and conventional PCR with different immunocapture formats and application of the technique to field samples

6.1. *Introduction*

The amplification of nucleic acid isolated from target pathogens from feces for PCR has been extremely difficult due to coextraction of inhibitors such as blood, bile salts, complex carbohydrates and other plant components. All of these factors may interfere with amplification and solutions were diluted to eliminate this problem (Chui *et al.*, 2004). Another problem associated with a bovine fecal specimen is its complexity and heterogeneous nature and *M. avium* subsp *paratuberculosis* (*Map)* cells clump in the feces. Consequently this presents a major challenge in purifying the DNA from *Map* in bovine feces, especially when the bacterial load is low. In Chapter 5, we have presented a combined method using a shaking protocol in the presence of 0.6% SDS, followed by two washes in water and with a final suspension of the pellet in PBS. This therefore helped to dissociate the bacterial clumps and to facilitate binding of *Map* to IgY immobilized on the beads. This method not only selects the organism but provides a means to concentrate it.

In previous chapters, conventional PCR using gel-based detection was used to determine the sensitivity and specificity of the assay. It is a qualitative assay assessing the presence of *Map* in specimens such as milk, bovine feces and tissues. However, the PCR cycling time is long and requires post-PCR manipulation for the detection of the

amplicons. Many laboratories lacking space to physically separate work areas can risk post-amplification contamination.

Real-time PCR is based on the detection of the first significant increase in fluorescence above a threshold level for a known set of standards. By analyzing the crossing point of the specimen against the known standards, real-time PCR has the potential of determining the initial level of nucleic acid by extrapolation to estimate the microbial load of the specimen.

In recent years, real-time assays have been developed for the detection of mycobacterial species (Fang *et al.*, 2002; Miller *et al.*, 2002; O'Mahony and Hill 2002, 2004). With advancement in amplification using real-time, the volume of target DNA required is reduced and it is not necessary to run the amplicons in gels for analysis. We compared conventional heat block PCR to a commercial real-time Light cycler PCR assay (RealArt™ *M. avium* subsp *paratuberculosis*, artus biotech USA, Inc CA), and an in-house real time assay using SYBR Green with melting curve analysis.

6.2 Materials and methods

6.2.1 *Preparation of Map in bovine feces*

Map ATCC strain 19698 was grown in Middlebrook 7H9 broth at pH 7.0 (Difco Laboratories, Detroit, Michigan), supplemented with 2% Middlebrook ADC (Becton Dickinsion Microbiology Systems, Sparkes, Maryland), 0.5% Tween 80 (Sigma Chemical Co., St. Louis, Missouri), 0.04% Mycobactin J (Allied Monitor, Fayette, Missouri) and incubated at 35°C for 2 to 3 weeks. Cells were harvested by

centrifugation at 1500 x *g* for 15 min. The pellet was washed twice with 5 ml of phosphate-buffered saline, pH 7.2 (PBS) and adjusted to different concentrations of cells based on the value of 1 OD_{550nm} = 2 x 10^6 to 10^7 cells/ml (Qiagen News, Issue #1, 1996; Qiagen, Mississauga, ON). The dilutions of cells included were 10^1, 10^2, 10^3, 10^4 and 10^5 cells/ml. The bovine feces used for the spiking experiment was provided by Agri-Food Laboratories Branch, Alberta Agriculture, Food and Rural Development. These fecal samples were previously tested negative for *Map* by standard culture method and kept frozen at –70ºC. The preparation of bovine feces spiked with *Map* cells was made according to the procedure described in Chapter 5, section 5.2.5.

6.2.2 *Capture assays by IMS technique*

The immunocapture assays were performed in 4.0 ml polystyrene tubes coated with *Map* IgY, by liquid capture or direct capture with MagaCell™-IgY in a 1.5 ml microcentrifuge tube. Five milliliter polystyrene tubes were coated with 1 ml of each of the dilutions (1:100, 1:200, 1:400 and 1:500) of IgY preadsorbed with *M. avium* resuspended in sodium carbonate (0.14mM)-sodium bicarbonate buffer (3.5mM) at pH 9.6. After 24 h incubation at 4ºC, the antibody solution was removed and 3 ml of Phosphate Buffer Saline with 0.05% Bovine serum albumin (PBS-0.05% BSA) at pH 8.2 was added to the tubes and the tubes were placed on a mixer (MyLab ™ Rotamix SLRM1, Seoulin, MJSBiolynx Inc., Brockville ON) for 5 min before the removal of the wash buffer. This washing procedure was repeated five times. One gram of bovine feces was spiked with 200 µl of *Map* cells at the concentration of 10^5/ml. This fecal

suspension was treated with 0.6% SDS as described in chapter 5, section 5.2.5 and 200 µl was added to the IgY coated tubes and incubated at 23°C for 30 min in a shaking water bath (NesLab, VWR International, Mississauga, ON) set at the speed of 5. After the incubation, the fecal cell suspension was removed and the tubes were washed 5 times with 1 ml of PBS. Two hundred microliters of 0.1 % SDS was added to the tube and hold at 23°C for 15 min and then removed from tube and used for DNA extraction by the MagaZorb® DNA kit (Cortex BioChem) with a semi-automated extractor (KingFisher, Thermo Electron, Bioscience Technologies) will subsequently be referred as the KingFisher extractor. All solutions were provided in the DNA extraction kit (Cortex BioChem) except the elution buffer (12mM Tris-HCl buffer at pH 7.4). Briefly, 20 µl of PK solution was added to 200 µl of the supernatant followed by 200 µl of Lysis Buffer, mixed by vortex for 10 sec, hold at 56°C for 10 min followed by adding to the first well of the extractor trough (KingFisher extractor). This well also contained 500 µl of binding buffer and 20 µl of MagaZorb® particles. In the adjacent second and third wells, 1 ml of Washing Buffer was added and followed by 200 µl of 12 mM Tris-HCl buffer at pH 7.4 as DNA elution buffer in the fourth well. The extractor was programmed as specified by the established protocol from Cortex BioChem. For control, same concentration of cells without feces was used for capture and DNA was extracted as described above. DNA was also extracted directly from cells and bovine feces spiked with cells without capture for comparison.

For liquid capture, 200 µl of each of the bovine feces spiked with different concentration of cells (10^1, 10^2, 10^3, 10^4 and 10^5 cells/ml) was added to a 1.5 ml

centrifuge tube containing *Map* IgY at a concentration of 1:500 dilution in a total volume of 1 ml in PBS. This suspension was held at 23°C for 30 min and followed by adding 2 µl of MagaBeads™ rabbit anti-chicken IgG (Cortex BioChem) for 30 min at the same temperature as specified by the manucfacturer. The beads were then washed 5 x with 12mM Tris-HCl, pH7.2 and 200ul of 1% SDS was added to the beads and the reaction was held at 23°C for 15 min and then removed from the tube by placing the tube in the Dynal MPC®-S unit (Dynal Biotech LLC, Brown Deer, WI). The supernatant separated from the beads was used for DNA extraction by the KingFisher extractor with MagaZorb® DNA kit (Cortex BioChem). For direct capture, 10 µl of MagaCell™-IgY beads was added to 1.5 ml microcentrifuge tubes containing 200 µl of the bovine fecal suspension spiked with *Map* cells pretreated with 0.6% SDS as described above in a total volume of 1 ml in PBS. After the capture, the beads were washed 5 x with 12mM Tris-HCl buffer at pH 7.4 and followed by 200 µl 1% SDS treatment and DNA extraction was carried out using the KingFisher extractor as described above. Direct extraction of DNA from cells in the presence of bovine feces were also compared using 2 commercial kits, MagaZorb® DNA isolation kit (Cortex BioChem) and QIAamp DNA stool mini kit (Qiagen Inc., Mississauga, ON). The following flowchart (Figure 6.1) shows immunocapture assay and different types of extraction of DNA for PCR.

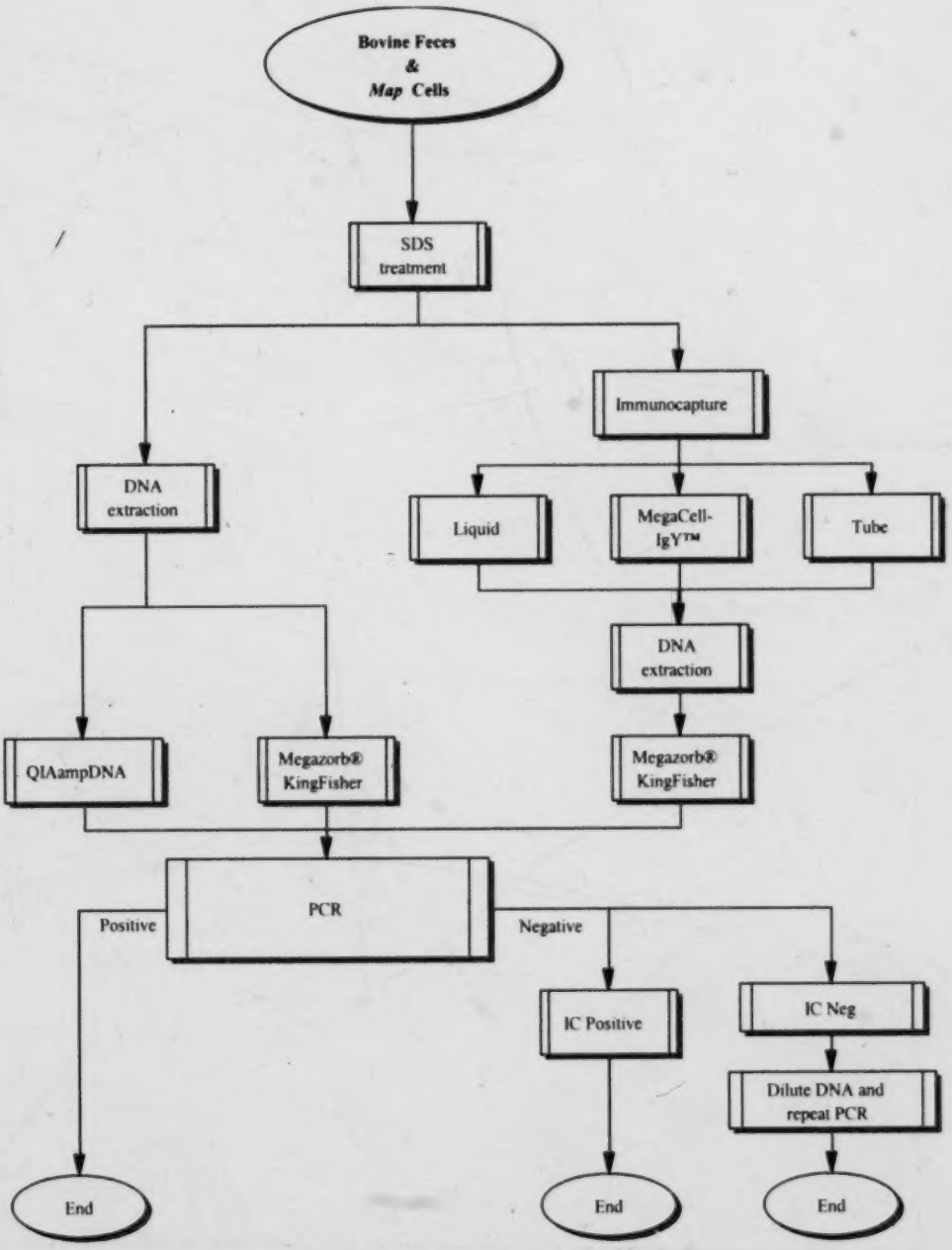

Figure 6.1 Flowchart showing immunocapture and direct extraction of DNA for PCR

6.2.3 *Conventional culture and capture culture of fecal samples*

Frozen bovine fecal specimens stored at –80°C were thawed at 4°C over night and approximately 1 g of the sample was weighed and added to a 60 ml sterile polypropylene centrifuge bottle with O-ring caps (Starplex Scientific Inc., Etibicoke, ON) and 35 ml of water was dispensed in the bottle and shaken on a mechanical shaker (Eberback model 6000, Ann Arbor, Michigan) for 30 min. The suspension was held at 23°C for 45 min and 25 ml was poured into a centrifuge tube (50 ml polypropylene centrifuge tube, VWR International Ltd. Mississauga, ON) and centrifuged at 1850 x *g* for 30 min. The pellet was resuspended in 25 ml of HPC-BHI (1-Hexadecylpyridium chloride Brain Heart Infusion broth) by using a vortex mixer. The tube was held at 37°C for 24 h. After this incubation, the suspension was centrifuged at 1850 x *g* for 30 min and the supernatant was removed. The pellet was resuspended in 1.2 ml of antibiotic-HPC-BHI (antibiotic-1-Hexadecylpyridium chloride Brain Heart Infusion broth) containing Amphotericin (50mg/l), Vancomycin (100 mg/l), Nalidixic Acid (100 mg/l) and this suspension was mixed by vortex and then held for 24 h at 37°C. Following incubation, the tube was removed and mixed by vortex for 2 min and 200 µl of suspension was inoculated into each of the 4 HEYM (Herrold's Egg Yolk medium with mycobactin) slants and 1 HEYMX (Herrold's egg yolk medium without mycobactin) slant and incubated at 36°C. The tubes were checked at 6 to 8 weeks, 10 to 12 weeks and a final check after 16 weeks post inoculation.

For the immunocapture assay, one gram of frozen feces was thawed overnight at 4°C and approximately 1 g of the sample was weighed and added to a conical centrifuge

tube containing 24 ml of 0.6% (w/v) SDS in water. The suspension was mixed by vortex for 1 min and was placed on a horizontal shaker (Eberback model 6000, Ann Arbor, Michigan) for 30 min set on low speed. After 30 min of standing at 23°C, to allow the particulate matter to settle, 20 ml of the supernatant was removed and centrifuged at 1850 x *g* for 30 min. The supernatant was discarded and 24 ml of sterile, purified water was added to the pellet, mixed by vortex for 1 min and centrifuged at 1850 x *g*. The supernatant was discarded and the pellet was resuspended in 1 ml of PBS, pH 7.4. An aliquot of 200 µl was added to 1.5 microcentrifuge tube and 10 µl of MagaCell™-IgY beads and PBS were added to a final volume of 1 ml. After the immunocapture, the tube was placed in the MPC®-S unit (DyNal Biotech LLC. WI) to separate the beads from the supernatant. The beads were washed 5 X with 12 mM Tris –HCl buffer at pH 7.4 and resuspended in 1000 µl of antibiotic-HPC-BHI and held at 23°C for 5 h. After this holding time, the tube was placed in the MPC®-S unit (DyNal Biotech LLC.), and the solution was removed and the beads were suspended in 1.2 ml of PBS and 200 µl of the suspension was added to each of 4 HEYM (with mycobactin) and 1 HEYMX (without mycobactin) tube for culture of *Map*. The tubes were checked for growth at 6 to 8 weeks, 10 to 12 weeks and finally after 16 weeks. Field strains, 11992, EA4146, 12258 and reference strain ATCC 19698 were included in the study.

6.2.4 *Polymerase chain reaction and detection*

DNA extracted from the different capture assays was used as target in conventional and real-time PCR. The primers for the conventional PCR and the condition were described previously in Chapter 3, section *3.2.5.* Detection of the amplicons was done by agarose gel electrophoresis. Real-time PCR using RealArt *M. paratuberculosis*™ (artus biotech, USA Inc.) amplification for *Map* was performed using Roche LightCycler™ (Roche Diagnostics, QC) Version 1.0, and the protocol was carried out according to the manufacturer's instructions. For both conventional and commercial real-time PCR assays, internal controls were added to monitor the inhibition of amplification. If the internal control failed to amplify along with the target, this indicated the presence of inhibitors that interfered with the PCR assay. The following published PCR primers directed to IS*900* (O'Mahony and Hill, 2002) designated as P90 and P91 were used for the in-house real-time assay with SYBR green (Roche Diagnostics, QC). The analysis was determined by the melting curve. Sequences of the PCR primers were as follows: P90: 5'-GAA GGG TGT TCG GGG CCG TCG CTT AGG-3' and P91: 5'-GGC GTT GAG GTC GAT CGC CCA CGT GAC-3'. PCR was performed using the Roche LightCycler™ (Roche Diagnostics, QC) Version 1.0 with the following parameters: 2 min at 95°C, 45 cycles of amplification with each cycle 0 s at 95°C, 5 s at 53°C and 10 s at 72°C. DNA was denatured from hybridization probes as follows: 0s at 95°C, 15 s at 45°C and 0 s at 95°C with 0.1°C /s slope and continuous acquisition. The final cooling step was for 30 s at 40°C.

6.2.5. *Field trial with real bovine feces*

The following fecal specimens were selected for the study: 44 positive fecal samples, 50 negative fecal samples from a *Map*-negative herd and 61 negative specimens from a *Map*-positive herd. All of the specimens were subjected to 0.6% SDS treatment as described in Chapter 5, section 5.2.5 and captured with 10 µl of MagaCell™-IgY, extracted using MagaZorb® DNA isolation kit (Cortex BioChem) and PCR was performed using conventional and RealArt™ *M. paratuberculosis* LC PCR kit (artus biotech USA, Inc.). All of the specimens were provided by Agri-Food Laboratories Branch, Alberta Agriculture, Food and Rural Development, and were tested using the standard culture procedure (section 6.2.3). For culture after the capture assay by MagaCell™-IgY beads using immuno magnetic separation technique, the beads were treated and cultured as described in section 6.2.3.

6.3 Results

The cell concentration used for the immunocapture assays was 20,000 *Map* cells (ATCC 19698) reacting with different concentrations of antibody (1:100, 1:200, 1:400 and 1:500) coated on polystyrene tubes. A summary of the conventional PCR endpoint titration results is shown in Table 6.1. The data showed that the PCR detection level was not affected by different concentrations of IgY used for coating the tubes in immunocapture assays. The PCR endpoint titration was at 10^{-4} of the diluted DNA extracted from the immunocaptured *Map* cells. Similar results were observed (Table 6.2) when bovine feces was spiked with *Map* cells in the immunocapture assay but the

titer of the end point titration was reduced 100-fold. Therefore any of the examined dilutions was suitable for coating the tubes for immunocapture assay.

Without immunocapture, the PCR endpoint with *Map* cells was detected at 10^{-5} which was 10-fold higher than the endpoint from the immunocaptured cells. This difference is probably due to the presence of DNA in the lysed bacteria found in the cell suspension.

Table 6.1. PCR endpoint titrations using conventional PCR with DNA extracted from 20,000 *Map* cells captured by different concentrations of IgY-coated tubes. DNA was extracted by Kingfisher extractor and diluted ten-fold for the PCR assay.

Dilutions of DNA	**PCR results with DNA extracted from cells after captured by different dilutions of IgY**			
	1:100	**1:200**	**1:400**	**1:500**
10^{-1}	+	+	+	+
10^{-2}	+	+	+	+
10^{-3}	+	+	+	+
10^{-4}	+	+	+	+
10^{-5}	-	-	-	-

Table 6.2. PCR endpoint titrations using conventional PCR with DNA extracted from 20,000 *Map* cell suspension mixed with bovine feces captured by different concentrations of IgY-coated tubes. DNA was extracted by Kingfisher extractor and diluted ten-fold for the PCR assay.

Dilutions of DNA	**PCR results with DNA extracted from cells suspension mixed with bovine feces after captured by different dilutions of IgY**			
	1:100	**1:200**	**1:400**	**1:500**
10^{-1}	+	+	+	+
10^{-2}	+	+	+	+
10^{-3}	-	-	-	-
10^{-4}	-	-	-	-
10^{-5}	-	-	-	-

PCR inhibition was observed previously in Chapter 3 with manual extraction using MagaZorb® DNA isolation kit of *Map* cells in the presence of bovine feces. A 1:100 dilution of the DNA was required to alleviate this problem. However, using the KingFisher extractor (Thermo Electron, Bioscience Technologies), a 1:10 dilution of DNA was sufficient to remove inhibitory factors coextracted with the target DNA. The automated washing of the extracted material appeared to be more efficient for removal of inhibitors.

Real-time PCR with RealArt™ *M. paratuberculosis* LC kit was performed in parallel with conventional PCR and the results are shown in Figures 6.1, 6.2 and 6.3. In Figure 6.1, a comparison of the PCR results was made between *Map* cells and *Map* cells in the presence of bovine feces. Sigmoidal curves of fluorescence were observed with dilutions of extracted DNA from 10^{-1} to 10^{-5} (Figure 6.1A) used as target for amplification from *Map* cells. Similar curves were observed with diluted DNA from 10^{-1} to 10^{-2} extracted from *Map* cells spiked with bovine feces (Figure 6.1B). In the presence of immunocapture using 1:100 or 1:500 dilutions of IgY coated tubes with *Map* cells, sigmoidal curves of fluorescence were observed with dilutions of DNA from 10^{-1} to 10^{-4} (Figure 6.2A) and similar curves were seen only from 10^{-1} to 10^{-2} with bovine feces spiked with *Map* cells (Figure 6.2B). Identical results were observed with 1:200 and 1:400 dilutions of IgY coated tubes. Dilutions of different IgY used for the immunocapture assays did not affect the outcome of the real-time PCR detection level but it was the presence of the bovine feces that played a role in the end result. Internal controls (IC) included in the RealArt™ *M. paratuberculosis* LC kit was incorporated

into each PCR capillary tube and no PCR inhibition was observed with DNA extracted from *Map* cells or *Map* cells after immunocapture.

No Capture

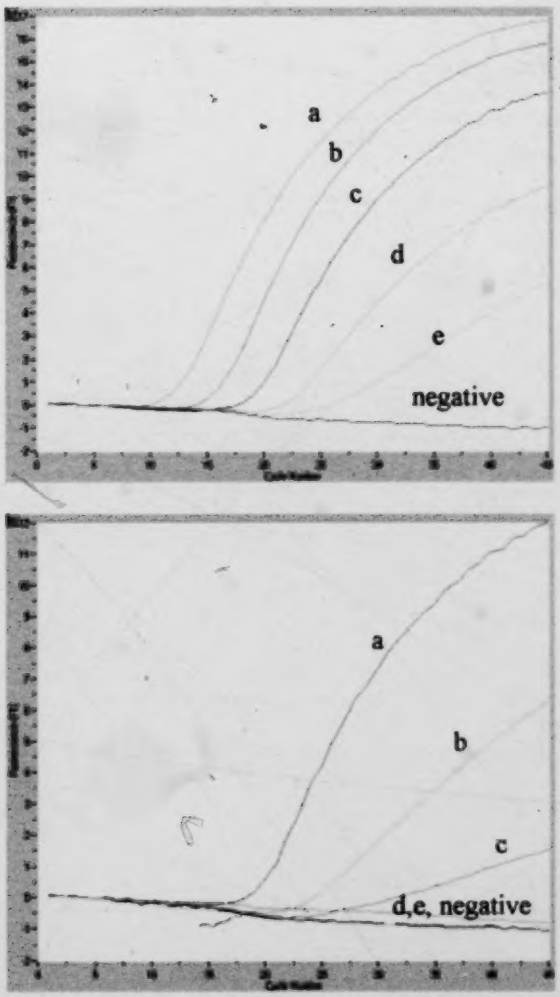

Figure 6.1. Amplification plots (fluorescence versus cycle number) of different dilutions of DNA extracted from 20,000 *Map* cells. In panel A, DNA was extracted from cells and in panel B, DNA was extracted from bovine feces spiked with cells; a to e are dilutions of DNA extracted from cells using the KingFisher extractor with MagaZorb® DNA isolation kit. a:10^{-1}, b:10^{-2}, c:10^{-3}, d:10^{-4}, e:10^{-5}.

Capture 1/100

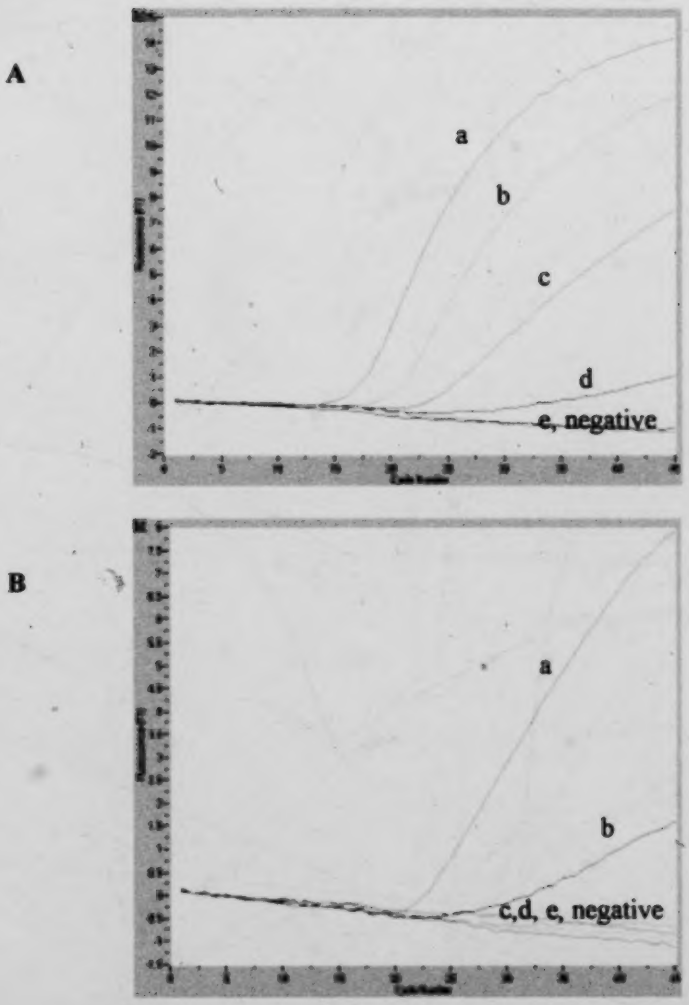

Figure 6.2. Amplification plots (fluorescence versus cycle number) of different dilutions of DNA extracted from 20,000 *Map* cells captured by 1:100 dilution of IgY coated polystyrene tubes; DNA was extracted using the KingFisher extractor with MagaZorb® DNA isolation kit. Extracted DNA was diluted from 10^{-1} to 10^{-5}; a:10^{-1}, b:10^{-2}, c:10^{-3}, d:10^{-4}, e:10^{-5}. In panel A, DNA was extracted from the captured cells and in panel B, DNA was extracted from bovine feces spiked with Map cells after capture.

Capture 1/500

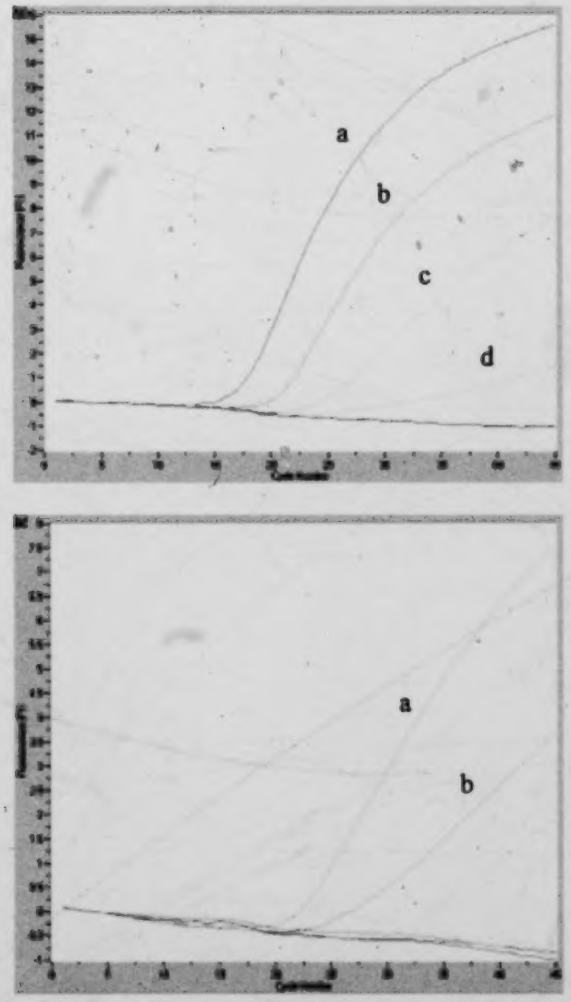

Figure 6.3. Amplification plots (fluorescence versus cycle number) of different dilutions of DNA extracted from 20,000 *Map* cells captured by 1:500 dilution of IgY coated polystyrene tubes; DNA was extracted using the KingFisher extractor with MagaZorb® DNA isolation kit. Extracted DNA was diluted from 10^{-1} to 10^{-5}; a:10^{-1}, b:10^{-2}, c:10^{-3}, d:10^{-4}, e:10^{-5}. In panel A, DNA was extracted from captured cells and in panel B, DNA was extracted from bovine feces spiked with *Map* cells after capture.

Results of the comparison between liquid, tube and MagaCell™-IgY bead immunocapture assays by real-time and conventional PCR are illustrated in Table 6.3. With real-time PCR detection using commercial, and in-house assays with liquid and MagaCell™-IgY capture, the endpoint detection for both types of immunocapture was at 1000 *Map* cells/g of spiked bovine feces. However, there was a 100-fold drop in the titer to 100,000 cells/g when IgY was immobilized onto the tube surface. This reduction of sensitivity was most likely caused by the IgY coated surface area of the tube. In both liquid and the MagaCell™-IgY capture, the beads were suspended in a liquid medium with shaking and this may have allowed a better chance of binding of the IgY to the target organisms. Furthermore, bovine feces is a very complex mixture, non-specific binding of the other materials would mask the IgY coated on the surface of the tubes and reduce the chance of IgY binding to *Map* cells. The data in Table 6.3 also showed that PCR endpoint titration was identical with the conventional PCR assays and the more convenient real-time assay.

A comparison of the detection level between the DNA manually extracted by the QIAamp DNA stool mini kit (Qiagen) and MagaZorb® DNA isolation kit (Cortex BioChem) by the KingFisher extractor is shown in Table 6.3. The results showed that the QIAamp DNA stool mini kit removed the nonspecific inhibitors and no dilution of the extracted DNA was required for PCR. Consequently, the PCR endpoint result was comparable to the endpoint of the immunocaptured assay by the liquid format or direct capture with MagaCell™-IgY. With direct extraction of bovine feces spiked with *Map* cells using the MagaZorb® DNA isolation kit by KingFisher extractor, a 1:10 dilution

was required to remove the inhibitors and thus reduced the PCR endpoint detection level by 10-fold as compared to using the QIAamp DNA stool mini kit (Qiagen) for extraction.

Table 6.3 PCR endpoint titration with extracted DNA from bovine feces spiked with *Map* cells in the presence or absence of immunocapture. A: Liquid capture: 1:500 dilution of IgY was added to bovine feces spiked with different *Map* cell concentrations and followed by a secondary capture using rabbit anti-chicken IgG Maga™ beads. B: immunocapture was performed using 1:100 dilution of IgY-coated tubes; C: direct capture with MagaCell™-IgY; D: direct extraction of DNA from samples and DNA was diluted 1:10 for PCR; DNA extractions from A to D were performed using MagaZorb® DNA isolation kit by the KingFisher. E: Direct extraction of samples using QIAamp DNA stool mini kit. Positive PCR results: + and negative PCR results:

Cells /ml	Real-time PCR										Conventional PCR				
	RealArt™ *M. paratuberculosis*					In-house SYBR green									
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1000	+	-	+	-	+	+	-	+	-	+	+	-	+	-	+
10000	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+
100000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

The times for completion based on 10 samples of bovine feces spiked with *Map* cells in the presence of absence of immunocapture in conjunction with real-time PCR are shown in Table 6.4. The most time efficient method of extracting DNA from *Map* in fecal samples was by direct extraction using the MagaZorb® DNA isolation kit with Kingfisher extractor. However, inhibition was still problematic and 1:10 dilution was required to achieve amplification and thus resulted in reduce sensitivity of the PCR assay. This is extremely important especially when the number of *Map* cells are low in the tested sample and a drop in sensitivity would generate a false negative result. Direct DNA extraction of *Map* cells in bovine feces by QIAamp DNA stool mini kit required the most time and was extremely laborious compared with the Kingfisher extractor in the presence or absence of immunocapture. Furthermore, there was one step in the procedure that required the addition of a solid inhibitor remover into the suspension of cells. The size of this inhibitor remover was greater than the circumference of the 1.5 ml micro centrifuge tube. Consequently, additional manipulation was required and this step can easily be the cause of contamination due to carry over from one tube to another. It is major concern for a diagnostic test. Comparison of standard culture and immunocapture with 10 µl of MagaCell™-IgY under optimized conditions at 23°C for 30 min was studied. At the concentration of 1000 cells in the presence of bovine feces using approximately 200 cells for the culture, only 5 colonies were detected /slant and with bovine feces spiked with 10,000 cells, 27 colonies were detected/slant. The colony counts of the culture results were average of the counts with all the strains tested.

Table 6.4 Comparison of time requirement based on 10 samples for extracting DNA from bovine feces spiked with *Map* cells coupled with real-time PCR with or without immunocapture.

Types of extraction coupled with real-time PCR	**Hands-on time (min)**	**Completion time (min)**
Capture with MagaCell™-IgY/ Kingfisher extraction with MagaZorb® DNA extraction kit	75	200
Direct extraction using Kingfisher with MagaZorb® DNA extraction kit	40	85
Direct extraction using QIAamp DNA stool mini kit	180	195

Samples from different farms from Alberta were selected for the field trial study and the results of conventional culture, culture after immunocapture with MagaCell™-IgY and PCR after immunocapture with MagaCell™-IgY using conventional and real-time amplification are shown in Table 6.5.

From the *Map*-positive farms, 44 samples previously identified as *Map*- positive by conventional culture by Agri-Food Laboratories Branch, Alberta Agriculture, Food and Rural Development were randomly selected. Immunocapture-culture found 27 of these samples positive. Conventional PCR after immunocapture, identified 34 of these samples as positive, 10 negative and inhibition was found in 16 samples initially but was resolved after a 1/10 dilution of the extracted DNA was re-tested by PCR. Immunocapture followed by real-time PCR identified 39 as positive and 5 as negative. All 50 negative samples from farms that had cattle which tested positive for *Map* were negative in all techniques tested.

Sixty-one samples from *Map* negative farms, fifty-nine out of sixty-one were culture-negative after immunocapture and the remaining two had to be rejected because of fungal contamination. In the PCR assay after immunocapture with samples from the *Map*-negative farm, inhibition was found in three samples that led to failure of the PCR. The other fifty-eight samples were all *Map* negative by PCR. Real-time amplification was performed using the commercial assay, RealArt™ *M. paratuberculosis* LC PCR kit and in-house assay with SYBR green with melting curve analysis on all of the field samples. There was no discordant result with conventional PCR and culture on negative samples found in the positive and negative farms.

More positive samples from the positive farms were identified using real-time PCR compared with conventional PCR (Table 6.5). Thirty-nine out of forty-four positive *Map* fecal samples were identified by real-time PCR and the remaining five samples experiencing no problem with inhibition but they were negative for *Map*. Since there was no internal control for the in-house real-time SYBR green assay with melting analysis, failure of amplification due to inhibition would not be identified.

Table 6.5. Comparison of different detection techniques for *Map* in bovine feces. All of these samples were subjected to 0.5% SDS treatment before using direct immunocapture with MagaCell™-IgY beads. DNA was extracted by manual extraction with MagaZorb® DNA isolation kit, conventional PCR and real-time PCR (RealArt ™ *M. paratuberculosis* LC PCR kit and in house SYBR green assay with melting curve analysis) were performed.

n* is the total number of random bovine fecal samples selected from all farms combined

Types of assays	Positive farms n*=44	Positive farms n=50	Negative farms n=61
Conventional culture	44/44 positive (100%)	50/50 negative (100%)	61/61 negative (100%)
Capture culture	27/44 positive (61.4%)	50/50 negative (100%)	59/61 negative 2/61 fungal contamination - no result (98.3%)
Capture Conventional PCR results including all samples that required dilutions to remove inhibition	34/44 positive (77.3%) 10/44 negative	50/50 negative (100%)	61/61 negative (100%)
Capture Real-time PCR	39/44 positive (88.6%) 5/44 negative	50/50 negative (100%)	61/61 (100%)

Based on conventional culture results, the field specimens examined in this chapter were divided into 2 categories and Table 6.6 is a summary showing sensitivities of real-time PCR, conventional PCR and culture after immunocapture with MagaCell™-IgY was applied to the tested samples. The first group consisted of specimens that gave heavy to medium growth characteristics on HEYM culture slants with counted *Map* colonies greater than 50. The second group were specimens that have light to scant growth on HEYM culture slants indicating there were only less than 50 colonies counted. There were eighteen samples in the heavy to medium growth category. With herds that are medium to heavy shedders, the sensitivities of capture PCR using real-time PCR (RealArt ™ *M. paratuberculosis* LC PCR kit and in-house SYBR green assay), conventional PCR and capture culture were 94.4%, 88.9% and 94.4% respectively. Real time assays and immunocapture culture missed one positive sample that was previously identified by conventional culture. In immunocapture with conventional PCR, initially only eleven out of eighteen samples (61.1%) were identified and there were six samples in this category showed inhibition with PCR. Upon 1:10 dilution of the extracted DNA, five samples turned positive and gave a total of sixteen positive samples and increased the positivity rate to 88.9%.

For the specimens that gave light or scant growth of *Map* from conventional culture, eight out of twenty-six samples (30.8%) were detected positive by conventional PCR after capture and another ten samples turned positive after a 1:10 dilution of the extracted DNA was amplified giving a total of 18 positive samples; increasing the percentage of positivity to 69.2. There were eight samples were misidentified as

negative. Result from real-time capture PCR in this category was 84.6%. The number of positive immunocapture culture samples identified in this category was ten out of twenty six with a detection rate of 38.5%. The captured culture result was drastically lower compared with the conventional culture method in the category of light to scant growth. This observation may due to loss of organisms through the extensive protocol of 0.1% SDS treatment in preparation for the immunocapture protocol. Furthermore, the washing steps of the immunocapture beads were tedious and difficult at times because in the fecal specimens, there were fecal materials that were attracted along with the beads to the Dynal MPC®-S unit (Dynal Biotech LLC) unit. It was diffcult to remove these non-specific materials and if extreme care was not taken, the beads could easily be lost during the extensive washing steps. The immunomagnetic beads with the bound *Map* cells could easily be trapped with the bovine feces and this may account of the low recovery of the organisms by capture-culture especially when the bacterial load was low in the specimens tested. There were also additional steps such as washing and antibiotic treatment of the captured cells, all these addional procedures that may account for the loss of *Map* cells resulted in the drop in the sensitivity with capture culture compared with the conventional culture method.

Table 6.6. Comparison of detection techniques after immunocapture with MagaCell™-IgY based on colony counts of *Map* cells. The types of growth on slants were previously determined by conventional culture method. Heavy growth to moderate growth: > 50 colonies. Light to scant: < 50 colonies. Inhibition to PCR was detected by the presence of the failure to amplification of the internal control present in the individual reaction tube in the RealArt ™ *M. paratuberculosis* LC PCR kit and in-house and the conventional PCR.

	Culture	Immunocapture with culture	Immunocapture with conventional PCR	Immunocapture with real-time PCR
Moderate to heavy growth	18/18 100%	17/18 94.4%	16/18 88.9% inhibition: 6 negative: 2	17/18 94.4% inhibition: 0 negative: 1
Scant to light growth	26/26 100%	10/26 38.5%	18/26 69.2% inhibition: 10 negative: 8	22/26 84.6% inhibition: 0 negative: 4
Overall	44/44 100%	27/44 61.4%	34/44 77.3%	39/44 88.6%

6.4 Discussion

Use of different types of immunocapture assays followed by real-time and conventional PCR comparison has not been previously reported. In this study, IgY immobilized on a matrix such as magnetic beads gave better binding of the antibody to the target organism than a solid phase, such as a tube surface. It did not matter whether the immunocapture assay was done using the IgY in the suspension followed by a secondary capture with a bead matrix or by direct immunocapture assay by MagaCell™-IgY.

Amplification tests such as PCR or nucleic acid sequenced-based amplification (NASBA) have been used (Bauerfeind *et al.*, 1996; Secott *et al.*, 1999; Van der Vliet *et al.*, 1993) to improve the turn-around time of PCR but the major problem is the presence of inhibitory factors that are coextracted with the target DNA. Using an immunocapture assay, the problem of inhibition can be circumvented and this technique also concentrates the target organisms during the immunocapture process (Djonne *et al.*, 2003; Grant *et al.*, 2000; Islam and Lindberg, 1992; Widjojoatmodjo *et al.*, 1992; Zhang and Weintraub, 1998).

Real-time PCR has a better sensitivity than conventional PCR when field samples were examined and this was not observed in chapter 5 when bovine feces was spiked with *Map* cells. The overall detection rates for immunocapture followed by real-time PCR, immunocapture followed by conventional PCR and immunocapture culture were 88.6%, 77.3% and 61.4%, respectively.

With immunocapture using MagaCell™-IgY beads, no inhibition was observed with spiked samples detected using conventional PCR. However, in the field trial with submitted field samples PCR inhibition was observed. This observation may be due to a number of reasons. In the field study, each fecal sample from individual animal was selected randomly from a designated farm as compared with pooled fecal samples previously tested negative for *Map* used for the spiking experiments. It seems reasonable to assume that the microflora of the individual animals different and as the number of different animals are tested different kinds of inhibitors would be encountered.

In the field study, more inhibition occurred in conventional PCR with fecal samples collected from positive cattle than from negative cattle. The bovine feces used for spiking experiments was identified as *Map*-negative samples by standard culture technique before use. The chronic inflammation present in positive cattle could generate a number of proteins and other substance not found in uninflamed cattle that would act as inhibitors. Although the capture assay was optimized for incubation time, temperature and extensive washing of the beads to eliminate as much as possible non-specific binding of other contaminating material before extraction of the DNA, there is no guarantee that 100 % of the inhibitory factors have been removed.

Similarly the comparison study examining different immunocapture formats with real-time and conventional PCR was repeated on four different occasions but the same pool of bovine feces was used for the spiking experiments. The sensitivity of the PCR assays after liquid and MagaCell™-IgY immunocapture was at a detection level of

1000 cell/g of bovine feces and the sensitivity is 10 fold higher as reported in Chapter 5. This increase in sensitivity in the spiked experiment most likely due to different lot suspension of cells used for the experiment.

In real-time PCR, the fluorescent signal was generated by the hybridization probe or by melting curve analysis with SYRA green. These technologies are much more sensitive than visual examination of the band by ethidium bromide under UV illumination. This may be responsible for the better performance of this technique. Nested or real-time PCR with a flourescent signals have been used to improve the sensitivity of the amplification assays (Collins *et al.*, 1993; Fang *et al.*, 2002). However, even though the primers used for the real-time and conventional PCR assays were selected from the target IS*900*, the sequences used, the amplification conditions, the size of the amplicons and analysis for the endpoint were different. The differences between the performance of the three techniques could be due to one or more of these as well.

The commercial assay for *Map* (RealArt ™ *M. paratuberculosis* LC PCR kit) incorporates a set of four standards to be included in the PCR run in which quantification of the DNA in the specimen could be calculated. However, we used the kit as a qualitative test and quantitation of the bacterial load was not calculated on the samples tested. An internal control was also included in each PCR reaction to monitor the effect of inhibitory factors. This internal control can also be spiked with the clinical samples and carry through the extraction procedure for monitoring the efficiency of the extraction protocol. The in-house real time assay used SYBR green with melting curve

analysis does not have the standards nor internal control and thus could not quantify the bacterial load in the specimens or detecting the presence of inhibition.

In this chapter, real-time PCR generated data with a high specificity by probe hybridization of melting curve analysis and a much faster turn-around time compared with conventional PCR that requires visualization of ethidium bromide stained amplicons under UV illumination. Submission of 50 fecal samples to a molecular diagnostic laboratory using immunocapture followed by automated extraction of DNA could be reported within a day shift of 8 h using real time amplification compared with two work shifts with conventional PCR. Either of these two assays has phenomenally improved the turn-around time compared with conventional culture which may require 8 to 20 weeks depending on the bacterial load of the samples. Furthermore, incorporation of a standard curve with different amounts of DNA for the real-time PCR assay can generate data for rough estimation of the bacterial load in the sample. All three assays are specific but the sensitivity depends on the load of *Map* present in the specimen. The sensitivity of the assay can be drastically improved if the sample volume is increased. This would be especially important to identify clinical specimens from the light shedders of *Map*. This capture PCR assay has significant potential for implementation in the screening for Johne's disease in cattle.

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6. General discussion and conclusions

In this study, purified IgY was used to develop a capture assay by immuno magnetic separation technology. By direct coating of purified IgY onto Magacell™ beads using 10µl under optimized condition at 23°C for 30 min, the detection level of *Map* by real-time PCR was at 1000 cells in the presence of one gram of bovine feces dispensed in 1 ml of PBS using 1:5 of this volume for the immunocapture assay. An improvement to this immunocapture assay would greatly improve if the amount of specimen used for the capture is increased to 10 g of bovine feces and dispensed into 250 ml of PBS follow with 0.6% SDS treatment and immunocapture using the total volume. Presently, there is a commercial instrument known as the Pathatrix system (Matrix Microscience, Inc., Golden CO). The Pathatrix system is a newly patented technology that depends on the use of specific antibody-coated paramagnetic particles to selectlively bind and purify the target organism from a complex matrix such as food or feces. An entire sample of 200 ml can be recirculated through a capture phase where the antibody-coated beads are immobilized. The capture beads are washed and ready to be processed either for culture, nucleic acid extraction for PCR or other identification assays. If the sample size is increased from the present study from 0.2 ml to 250 ml for the immunocapture assay using the Pathatrix system, it will definitely increase the detection level of *Map* especially for light shedders of *Map* at the early stage of the disease. Frontline automation with immunocapture and washing using the Pathatrix system, follows by automated extraction of DNA coupled with real-time PCR will

definitely increase the sensitivity and efficiency. This combination of technologies will allow high volume *Map* screening for Johne's disease.

Johne's disease occurs worldwide and has great economic impact especially on the cattle industry. The early control program for Johne's disease was based mainly on early culling of the infected animals (Kalis *et al.*, 1999). The outcome of such a control program was disappointing due to the lack of sensitivity in diagnostic tests to identify infected animals at the early stage of disease and farmers also have not been consistent in maintaining husbandry measures to prevent transmission of infection. A change of strategy has occurred through the use of vaccination. However, the vaccine did not eliminate the disease but it only reduces its severity and infected animals still shed *Map* in the feces and thus contaminating the environment. In 1999, the project known as "Preparation for the collective control of paratuberculosis in The Netherlands" was initiated (Groenendaal *et al.*, 2003). The objective of this project was to prepare a national Johne's disease control program with the aim to eradicate the disease and this project led to the implementation of the Dutch voluntary national Johne's disease control program in September 2000 (Groenendaal *et al.*, 2003). The focus was a fundamental change in the design of the control program from "test and cull" to "stepwise improvements of calf hygiene". This awareness of good husbandry measures on farmland would have greater impact on the eradication of Johne's disease.

The Alberta Johne's Disease Control Program (Alberta Agriculture, Food and Rural Development, {AAFRD} 2004) was initiated in 2001/2002 by Alberta Agriculture, Food and Rural Development in conjunction with Alberta Johne's Working

Group with members consisted of representatives from the ruminant livestock industries, the veterinary organizations and government. Presently, the control program is based on voluntary participation of the producers and the enrolment is through veterinarians accredited by the Alberta Johne's working group. For herds that have been enrolled in the program, a confidential code number would be assigned by the accredited veterinarian and samples would be submitted for testing at Alberta Agriculture, Food and Rural Development (AAFRD), a United States Department of Agriculture certified laboratory. There are two major goals for this program and they are to identify and categorize herds that are free of Johne's disease and also to educate the producers on the risk of infection associated with cattle movement especially in international trade.

There are four levels of certification associated with this control program (AAFRD, 2004). In order to attain a level I certification, the accredited veterinarian submits 30 randomly selected blood samples from eligible cattle (at least at the second lactation) for enzyme-linked immnosorbent assay (ELISA) testing and the result of individual animal has to be negative. However, if the result is positive, a fecal sample is submitted as a follow-up specimen for culture. If culture is negative, then the herd is awarded with a level I certification and will remain at this level between 6 to 18 months.

At level II certification, fecal samples are submitted for culture from eligible cattle in the herd. Samples are strategically pooled for fecal culture and all samples have to be tested negative for *Map*. The herd must remain at this level for 12 months before proceeding to the next level. From level II to level III certification, fecal samples are submitted and strategically pooled for culture and all the results have to be tested

negative. One year of waiting at level III is required before resubmission of fecal samples for level IV certification and all culture results have to be negative for the herd to be awarded for this level. The farmer can maintain level IV status with annual ELISA testing of 30 random blood samples from cattle of second lactation of greater and all submitted samples have to be tested negative by ELISA or confirmed negative on fecal culture.

The accredited training program of the veterinarians (AAFRD, 2004) includes information pertaining to the specifics of Johne's disease, Johne's Herd Status program and issues related to the control of this disease. These veterinarians are instrumental in conveying the information about Johne's disease, the availability of diagnostic tests and their limitation, the control program, benefits and commitment to the program. They also maintain records of submission for audit purposes and confidentiality of farm data using a coding system. There is no mandate on farmers' participation, however, providing certification of Johne's status of the herd would add value to trading of cattle in the market. The role of participant in this program is required to keep records of herd inventory and list of animals that are three years of age (second lactation) or older. The Alberta Johne's Working Group is to determine the working policy of the program, training and accrediting veterinarians, assigning code to accredited veterinarians, maintaining and registering the herd status of participating herds.

The Alberta Johne's Disease Control Program was driven by concerns over the prevalence of Johne's disease in Alberta. The present strategy is to have multiple testing of the herds to ensure the freedom of disease and the two tests available are ELISA and

fecal culture. Improvement for sensitivity and rapid turn-around time are the key issues in searching for an alternative method of testing that fulfill these criteria. Increasing the amount of bovine feces using the Pathatrix system for IgY immunocapture assay developed in this study, coupled with automated DNA extraction and real-time PCR, it will improve the current existing protocols (ELISA and fecal culture) for Alberta Johne's Herd Status Program. The implementation of such a sensitive diagnostic test along with rapid turn-around-time will enhance the surveillance program for Johne's disease. Continued education on the the awareness of Johne's disease with commitment to the control program along with good husbandry and control measures, Johne's disease can easily be controlled in the farms.

7.1 References

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